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(54) Title: METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

(57) Abstract: The present invention provides methods for attenuating gene expression in a cell using gene-targeted double stranded RNA (dsRNA). The dsRNA contains a nucleotide sequence that hybridizes under physiologic conditions of the cell to the nucleotide sequence of at least a portion of the gene to be inhibited (the "target" gene).

## *Methods and Compositions for RNA Interference*

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### Background of the Invention

“RNA interference”, “post-transcriptional gene silencing”, “quelling” — these different names describe similar effects that result from the overexpression or 10 misexpression of transgenes, or from the deliberate introduction of double-stranded RNA into cells (reviewed in Fire A (1999) *Trends Genet* 15:358–363; Sharp PA (1999) *Genes Dev* 13:139–141; Hunter C (1999) *Curr Biol* 9:R440–R442; Baulcombe DC (1999) *Curr Biol* 9:R599–R601; Vaucheret et al. (1998) *Plant J* 16:651–659). The injection of double-stranded RNA into the nematode *Caenorhabditis elegans*, for example, acts systemically 15 to cause the post-transcriptional depletion of the homologous endogenous RNA (Fire et al. (1998) *Nature* 391: 806–811; and Montgomery et al. (1998) *PNAS* 95:15502–15507). RNA interference, commonly referred to as RNAi, offers a way of specifically and 20 potently inactivating a cloned gene, and is proving a powerful tool for investigating gene function. But the phenomenon is interesting in its own right; the mechanism has been rather mysterious, but recent research — the latest reported by Smardon et al. (2000) *Curr Biol* 10:169–178 — is beginning to shed light on the nature and evolution of the biological 25 processes that underlie RNAi.

RNAi was discovered when researchers attempting to use the antisense RNA approach to inactivate a *C. elegans* gene found that injection of sense-strand RNA was 25 actually as effective as the antisense RNA at inhibiting gene function. Guo et al. (1995) *Cell* 81:611–620. Further investigation revealed that the active agent was modest amounts of double-stranded RNA that contaminate *in vitro* RNA preparations. Researchers quickly determined the ‘rules’ and effects of RNAi. Exon sequences are required, whereas introns and promoter sequences, while ineffective, do not appear to compromise RNAi (though 30 there may be gene-specific exceptions to this rule). RNAi acts systemically — injection into one tissue inhibits gene function in cells throughout the animal. The results of a variety of experiments, in *C. elegans* and other organisms, indicate that RNAi acts to destabilize cellular RNA after RNA processing.

The potency of RNAi inspired Timmons and Fire (1998 *Nature* 395: 854) to do a simple experiment that produced an astonishing result. They fed to nematodes bacteria that had been engineered to express double-stranded RNA corresponding to the *C. elegans* *unc-22* gene. Amazingly, these nematodes developed a phenotype similar to that of *unc-22* mutants that was dependent on their food source. The ability to conditionally expose large numbers of nematodes to gene-specific double-stranded RNA formed the basis for a very powerful screen to select for RNAi-defective *C. elegans* mutants and then to identify the corresponding genes.

Double-stranded RNAs (dsRNAs) can provoke gene silencing in numerous *in vivo* contexts including *Drosophila*, *Caenorhabditis elegans*, planaria, hydra, trypanosomes, fungi and plants. However, the ability to recapitulate this phenomenon in higher eukaryotes, particularly mammalian cells, has not be accomplished in the art. Nor has the prior art demonstrated that this phenomena can be observe in cultured eukaryotes cells.

### 15 Summary of the Invention

One aspect of the present invention provides a method for attenuating expression of a target gene in a non-embryonic cell suspended in culture, comprising introducing into the cell a double stranded RNA (dsRNA) in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the target gene.

Another aspect of the present invention provides a method for attenuating expression of a target gene in a mammalian cell, comprising

- (i) activating one or both of a Dicer activity or an Argonaut activity in the cell, and
- 25 (ii) introducing into the cell a double stranded RNA (dsRNA) in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the target gene.

In certain embodiments, the cell is suspended in culture; while in other embodiments the 30 cell is in a whole animal, such as a non-human mammal.

In certain preferred embodiments, the cell is engineered with (i) a recombinant gene encoding a Dicer activity, (ii) a recombinant gene encoding an Argonaut activity, or (iii) both. For instance, the recombinant gene may encode, for a example, a protein which includes an amino acid sequence at least 50 percent identical to SEQ ID No. 2 or 4; or be

defined by a coding sequence hybridizes under wash conditions of 2 x SSC at 22°C to SEQ ID No. 1 or 3. In certain embodiments, the recombinant gene may encode, for example, a protein which includes an amino acid sequence at least 50 percent identical to the Argonaut sequence shown in Figure 24.

5 In certain embodiments, rather than use a heterologous expression construct(s), an endogenous Dicer gene or Argonaut gene can be activated, e.g., by gene activation technology, expression of activated transcription factors or other signal transduction protein, which induces expression of the gene, or by treatment with an endogenous factor which upregulates the level of expression of the protein or inhibits the degradation of the  
10 protein.

In certain preferred embodiments, the target gene is an endogenous gene of the cell. In other embodiments, the target gene is a heterologous gene relative to the genome of the cell, such as a pathogen gene, e.g., a viral gene.

15 In certain embodiments, the cell is treated with an agent that inhibits protein kinase RNA-activated (PKR) apoptosis, such as by treatment with agents which inhibit expression of PKR, cause its destruction, and/or inhibit the kinase activity of PKF.

In certain preferred embodiments, the cell is a primate cell, such as a human cell.

In certain embodiments, the dsRNA is at least 50 nucleotides in length, and preferably 400-800 nucleotides in length.

20 Still another aspect of the present invention provides an assay for identifying nucleic acid sequences responsible for conferring a particular phenotype in a cell, comprising

- (i) constructing a variegated library of nucleic acid sequences from a cell in an orientation relative to a promoter to produce double stranded DNA;
- 25 (ii) introducing the variegated dsRNA library into a culture of target cells, which cells have an activated Dicer activity or Argonaut activity;
- (iii) identifying members of the library which confer a particular phenotype on the cell, and identifying the sequence from a cell which correspond, such as being identical or homologous, to the library member.

30

Yet another aspect of the present invention provides a method of conducting a drug discovery business comprising:

- (i) identifying, by the assay of claim 16, a target gene which provides a phenotypically desirable response when inhibited by RNAi;
- (ii) identifying agents by their ability to inhibit expression of the target gene or the activity of an expression product of the target gene;
- 5 (iii) conducting therapeutic profiling of agents identified in step (b), or further analogs thereof, for efficacy and toxicity in animals; and
- (iv) formulating a pharmaceutical preparation including one or more agents identified in step (iii) as having an acceptable therapeutic profile.

10 The method may include an additional step of establishing a distribution system for distributing the pharmaceutical preparation for sale, and may optionally include establishing a sales group for marketing the pharmaceutical preparation.

Another aspect of the present invention provides a method of conducting a target discovery business comprising:

- (i) identifying, by the assay of claim 16, a target gene which provides a phenotypically desirable response when inhibited by RNAi;
- 15 (ii) (optionally) conducting therapeutic profiling of the target gene for efficacy and toxicity in animals; and
- (iii). licensing, to a third party, the rights for further drug development of inhibitors of the target gene.

20 Another aspect of the invention provides a method for inhibiting RNAi by inhibiting the expression or activity of an RNAi enzyme. Thus, the subject method may include inhibiting the acitivity of Dicer and/or the 22-mer RNA.

Still another aspect relates to the a method for altering the specificity of an RNAi by modifying the sequence of the RNA component of the RNAi enzyme.

25 Another aspect of the invention relates to purified or semi-purified preparations of the RNAi enzyme or components thereof. In certain embodiments, the preparations are used for identifying compounds, especially small organic molecules, which inhibit or potentiate the RNAi activity. Small molecule inhibitors, for example, can be used to inhibit dsRNA responses in cells which are purposefully being transfected with a virus 30 which produces double stranded RNA.

The dsRNA construct may comprise one or more strands of polymerized ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. The double-stranded structure may be formed by a single self-complementary

RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The dsRNA construct may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that 5 nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. dsRNA constructs containing a nucleotide sequences identical to a portion of the target gene is preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by alignment 10 algorithms known in the art and calculating the percent difference between the nucleotide sequences. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript.

15 **Brief Description of the Drawings**

Figure 1: RNAi in S2 cells. a, *Drosophila* S2 cells were transfected with a plasmid that directs *lacZ* expression from the copia promoter in combination with dsRNAs corresponding to either human CD8 or *lacZ*, or with no dsRNA, as indicated. b, S2 cells were co-transfected with a plasmid that directs expression of a GFP-US9 fusion protein 20 (12) and dsRNAs of either *lacZ* or *cyclin E*, as indicated. Upper panels show FACS profiles of the bulk population. Lower panels show FACS profiles from GFP-positive cells. c, Total RNA was extracted from cells transfected with *lacZ*, *cyclin E*, *fizzy* or *cyclin A* dsRNAs, as indicated. Northern blots were hybridized with sequences not present in the transfected dsRNAs.

25 Figure 2: RNAi *in vitro*. a, Transcripts corresponding to either the first 600 nucleotides of *Drosophila cyclin E* (E600) or the first 800 nucleotides of *lacZ* (Z800) were incubated in lysates derived from cells that had been transfected with either *lacZ* or *cyclin E* (cycE) dsRNAs, as indicated. Time points were 0, 10, 20, 30, 40 and 60 min for *cyclin E* and 0, 10, 20, 30 and 60 min for *lacZ*. b, Transcripts were incubated in an extract of S2 30 cells that had been transfected with *cyclin E* dsRNA (cross-hatched box, below). Transcripts corresponded to the first 800 nucleotides of *lacZ* or the first 600, 300, 220 or 100 nucleotides of *cyclin E*, as indicated. Eout is a transcript derived from the portion of the *cyclin E* cDNA not contained within the transfected dsRNA. E-ds is identical to the dsRNA that had been transfected into S2 cells. Time points were 0 and 30 min. 35 c, Synthetic transcripts complementary to the complete *cyclin E* cDNA (Eas) or the final

600 nucleotides (Eas600) or 300 nucleotides (Eas300) were incubated in extract for 0 or 30 min.

Figure 3: Substrate requirements of the RISC. Extracts were prepared from cells transfected with *cyclin E* dsRNA. Aliquots were incubated for 30 min at 30 °C before the 5 addition of either the *cyclin E* (E600) or *lacZ* (Z800) substrate. Individual 20-µl aliquots, as indicated, were pre-incubated with 1 mM CaCl<sub>2</sub> and 5 mM EGTA, 1 mM CaCl<sub>2</sub>, 5 mM EGTA and 60 U of micrococcal nuclease, 1 mM CaCl<sub>2</sub> and 60 U of micrococcal nuclease or 10 U of DNase I (Promega) and 5 mM EGTA. After the 30-min pre-incubation, EGTA was added to those samples that lacked it. Yeast tRNA (1 µg) was added to all samples. 10 Time points were at 0 and 30 min.

Figure 4: The RISC contains a potential guide RNA. a, Northern blots of RNA from either a crude lysate or the S100 fraction (containing the soluble nuclease activity, see Methods) were hybridized to a riboprobe derived from the sense strand of the *cyclin E* mRNA. b, Soluble *cyclin-E*-specific nuclease activity was fractionated as described in 15 Methods. Fractions from the anion-exchange resin were incubated with the *lacZ*, control substrate (upper panel) or the cyclin E substrate (centre panel). Lower panel, RNA from each fraction was analysed by northern blotting with a uniformly labelled transcript derived from sense strand of the *cyclin E* cDNA. DNA oligonucleotides were used as size markers.

Figure 5: Generation of 22mers and degradation of mRNA are carried out by 20 distinct enzymatic complexes. A. Extracts prepared either from 0-12 hour *Drosophila* embryos or *Drosophila* S2 cells (see Methods) were incubated 0, 15, 30, or 60 minutes (left to right) with a uniformly-labeled double-stranded RNA corresponding to the first 500 nucleotides of the *Drosophila cyclin E* coding region. M indicates a marker prepared 25 by *in vitro* transcription of a synthetic template. The template was designed to yield a 22 nucleotide transcript. The doublet most probably results from improper initiation at the +1 position. B. Whole-cell extracts were prepared from S2 cells that had been transfected with a dsRNA corresponding to the first 500 nt. of the luciferase coding region. S10 extracts were spun at 30,000xg for 20 minutes which represents our standard RISC 30 extract<sup>6</sup>. S100 extracts were prepared by further centrifugation of S10 extracts for 60 minutes at 100,000xg. Assays for mRNA degradation were carried out as described previously<sup>6</sup> for 0,30 or 60 minutes (left to right in each set) with either a single-stranded luciferase mRNA or a single-stranded cyclin E mRNA, as indicated. C. S10 or S100 extracts were incubated with cyclin E dsRNAs for 0, 60 or 120 minutes (L to R).

35 Figure 6: Production of 22mers by recombinant CG4792/Dicer. A. *Drosophila* S2 cells were transfected with plasmids that direct the expression of T7-epitope tagged

versions of Drosha, CG4792/Dicer-1 and Homeless. Tagged proteins were purified from cell lysates by immunoprecipitation and were incubated with *cyclin E* dsRNA. For comparison, reactions were also performed in *Drosophila* embryo and S2 cell extracts. As a negative control, immunoprecipitates were prepared from cells transfected with a  $\beta$ -galactosidase expression vector. Pairs of lanes show reactions performed for 0 or 60 minutes. The synthetic marker (M) is as described in the legend to Figure 1. B. Diagrammatic representations of the domain structures of CG4792/Dicer-1, Drosha and Homeless are shown. C. Immunoprecipitates were prepared from detergent lysates of S2 cells using an antiserum raised against the C-terminal 8 amino acids of *Drosophila* Dicer-1 (CG4792). As controls, similar preparations were made with a pre-immune serum and with an immune serum that had been pre-incubated with an excess of antigenic peptide. Cleavage reactions in which each of these precipitates was incubated with an ~500 nt. fragment of *Drosophila* cyclin E are shown. For comparison, an incubation of the substrate in *Drosophila* embryo extract was electrophoresed in parallel. D. Dicer immunoprecipitates were incubated with dsRNA substrates in the presence or absence of ATP. For comparison, the same substrate was incubated with S2 extracts that either contained added ATP or that were depleted of ATP using glucose and hexokinase (see methods). E. *Drosophila* S2 cells were transfected with uniformly, 32P-labelled dsRNA corresponding to the first 500 nt. of GFP. RISC complex was affinity purified using a histidine-tagged version of D.m. Ago-2, a recently identified component of the RISC complex (Hammond et al., in prep). RISC was isolated either under conditions in which it remains ribosome associated (ls, low salt) or under conditions that extract it from the ribosome in a soluble form (hs, high salt)<sup>6</sup>. For comparison, the spectrum of labelled RNAs in the total lysate is shown. F. Guide RNAs produced by incubation of dsRNA with a Dicer immunoprecipitate are compared to guide RNAs present in a affinity-purified RISC complex. These precisely comigrate on a gel that has single-nucleotide resolution. The lane labelled control is an affinity selection for RISC from cell that had been transfected with labeled dsRNA but not with the epitope-tagged D.m. Ago-2.

Figure 7: Dicer participates in RNAi. A. *Drosophila* S2 cells were transfected with dsRNAs corresponding to the two *Drosophila* Dicers (CG4792 and CG6493) or with a control dsRNA corresponding to murine caspase 9. Cytoplasmic extracts of these cells were tested for Dicer activity. Transfection with Dicer dsRNA reduced activity in lysates by 7.4-fold. B. The Dicer-1 antiserum (CG4792) was used to prepare immunoprecipitates from S2 cells that had been treated as described above. Dicer dsRNA reduced the activity of Dicer-1 in this assay by 6.2-fold. C. Cells that had been transfected two days previously with either mouse caspase 9 dsRNA or with Dicer dsRNA were cotransfected with a GFP expression plasmid and either control, luciferase dsRNA or GFP dsRNA.

Three independent experiments were quantified by FACS. A comparison of the relative percentage of GFP-positive cells is shown for control (GFP plasmid plus luciferase dsRNA) or silenced (GFP plamsid plus GFP dsRNA) populations in cells that had previously been transfected with either control (caspase 9) or Dicer dsRNAs.

5       Figure 8: Dicer is an evolutionarily conserved ribonuclease. A. A model for production of 22mers by Dicer. Based upon the proposed mechanism of action of Ribonuclease III, we propose that Dicer acts on its substrate as a dimer. The positioning of the two ribonuclease domains (RIIIa and RIIIb) within the enzyme would thus determine the size of the cleavage product. An equally plausible alternative model could  
10      be derived in which the RIIIa and RIIIb domains of each Dicer enzyme would cleave in concert at a single position. In this model, the size of the cleavage product would be determined by interaction between two neighboring Dicer enzymes. B. Comparison of the domain structures of potential Dicer homologs in various organisms (*Drosophila* - CG4792, CG6493, *C. elegans* - K12H4.8, *Arabidopsis* - CARPEL FACTORY<sup>24</sup>,  
15      T25K16.4, AC012328\_1, human Helicase-MOI<sup>25</sup> and *S. pombe* - YC9A\_SCHPO). The ZAP domains were identified both by analysis of individual sequences with Pfam<sup>26</sup> and by Psi-blast<sup>28</sup> searches. The ZAP domain in the putative *S. pombe* Dicer is not detected by PFAM but is identified by Psi-Blast and is thus shown in a different color. For comparison, a domain structure of the RDE1/QDE2/ARGONAUTE family is shown. It  
20      should be noted that the ZAP domains are more similar within each of the Dicer and ARGONAUTE families than they are between the two groups. C. An alignment of the ZAP domains in selected Dicer and Argonaute family members is shown. The alignment was produced using ClustalW.

Figure 9: Purification strategy for RISC. (second step in RNAi model).

25       Figure 10: Fractionation of RISC activity over sizing column. Activity fractionates as 500KD complex. Also, antibody to dm argonaute 2 cofractionates with activity.

Figure 11-13: Fractionation of RISC over monoS, monoQ, Hydroxyapatite columns. Dm argonaute 2 protein also cofractionates.

Figure 14: Alignment of dm argonaute 2 with other family members.

30       Figure 15: Confirmation of dm argonaute 2. S2 cells were transfected with labeled dsRNA and His tagged argonaute. Argonaute was isolated on nickel agarose and RNA component was identified on 15% acrylamide gel.

Figure 16: S2 cell and embryo extracts were assayed for 22mer generating activity.

Figure 17: RISC can be separated from 22mer generating activity (dicer). Spinning extracts (S100) can clear RISC activity from supernatant (left panel) however, S100 spins still contain dicer activity (right panel).

Figure 18: Dicer is specific for dsRNA and prefers longer substrates.

5 Figure 19: Dicer was fractionated over several columns.

Figure 20: Identification of dicer as enzyme which can process dsRNA into 22mers. Various RNaseIII family members were expressed with n terminal tags, immunoprecipitated, and assayed for 22mer generating activity ( left panel). In right panel, antibodies to dicer could also precipitate 22mer generating activity.

10 Figure 21: Dicer requires ATP.

Figure 22: Dicer produces RNAs that are the same size as RNAs present in RISC.

Figure 23: Human dicer homolog when expressed and immunoprecipitated has 22mer generating activity.

15 Figure 24: Sequence of dm argonaute 2. Peptides identified by microsequencing are shown in underline.

Figure 25: Molecular characterization of dm argonaute 2. The presence of an intron in coding sequence was determined by northern blotting using intron probe. This results in a different 5' reading frame than that published genome sequence. Number of polyglutamine repeats was determined by genomic PCR.

20 Figure 26: Dicer activity can be created in human cells by expression of human dicer gene. Host cell was 293. Crude extracts had dicer activity, while activity was absent from untransfected cells. Activity is not dissimilar to that seen in drosophila embryo extracts..

25 Figure 27: An ~500 nt. fragment of the gene that is to be silenced (X) is inserted into the modified vector as a stable direct repeat using standard cloning procedures. Treatment with commercially available cre recombinase reverses sequences within the loxP sites (L) to create an inverted repeat. This can be stably maintained and amplified in an sbc mutant bacterial strain (DL759). Transcription in vivo from the promoter of choice (P) yields a hairpin RNA that causes silencing. A zeocin resistance marker is included to 30 insure maintenance of the direct and inverted repeat structures; however this is non-essential in vivo and could be removed by pre-mRNA splicing if desired. Smith, N. A. *et al.* Total silencing by intron-spliced hairpin RNAs. *Nature* 407, 319-20 (2000).

**Detailed Description of the Certain Preferred Embodiments*****I. Overview***

The present invention provides methods for attenuating gene expression in a cell 5 using gene-targeted double stranded RNA (dsRNA). The dsRNA contains a nucleotide sequence that hybridizes under physiologic conditions of the cell to the nucleotide sequence of at least a portion of the gene to be inhibited (the "target" gene).

A significant aspect to certain embodiments of the present invention relates to the demonstration in the present application that RNAi can in fact be accomplished in cultured 10 cells, rather than whole organisms as described in the art.

Another salient feature of the present invention concerns the ability to carry out RNAi in higher eukaryotes, particularly in non-oocytic cells of mammals, e.g., cells from adult mammals as an example.

As described in further detail below, the present invention(s) are based on the 15 discovery that the RNAi phenomenon is mediated by a set of enzyme activities, including an essential RNA component, that are evolutionarily conserved in eukaryotes ranging from plants to mammals.

One enzyme contains an essential RNA component. After partial purification, a multi-component nuclease (herein "RISC nuclease") co-fractionates with a discrete, 22- 20 nucleotide RNA species which may confer specificity to the nuclease through homology to the substrate mRNAs. The short RNA molecules are generated by a processing reaction from the longer input dsRNA. Without wishing to be bound by any particular theory, these 22mer guide RNAs may serve as guide sequences that instruct the RISC nuclease to destroy specific mRNAs corresponding to the dsRNA sequences.

25 The appended examples also identify an enzyme, Dicer, that can produce the putative guide RNAs. Dicer is a member of the RNase III family of nucleases that specifically cleave dsRNA and is evolutionarily conserved in worms, flies, plants, fungi and, as described herein, mammals. The enzyme has a distinctive structure which includes a helicase domain and dual RNase III motifs. Dicer also contains a region of homology to 30 the RDE1/QDE2/ARGONAUTE family, which have been genetically linked to RNAi in lower eukaryotes. Indeed, activation of, or overexpression of Dicer may be sufficient in many cases to permit RNA interference in otherwise non-receptive cells, such as cultured eukaryotic cells, or mammalian (non-oocytic) cells in culture or in whole organisms.

In certain embodiments, the cells can be treated with an agent(s) that inhibits the double-stranded RNA-dependent protein known as PKR (protein kinase RNA-activated). Double stranded RNAs in mammalian cells typically activate protein kinase PKR that phosphorylates and inactivates eIF2a (Fire (1999) *Trends Genet* 15:358). The ensuing 5 inhibition of protein synthesis ultimately results in apoptosis. This sequence-independent response may reflect a form of primitive immune response, since the presence of dsRNA is a common feature of many viral lifecycles. However, as described herein, Applicants have demonstrated that the PKR response can be overcome in favor of the sequence-specific RNAi response. However, in certain instances, it can be desirable to treat the 10 cells with agents which inhibit expression of PKR, cause its destruction, and/or inhibit the kinase activity of PKR are specifically contemplated for use in the present method. Likewise, overexpression of or agents which ectopically activate IF2 $\alpha$  can be used.

Thus, the present invention provides a process and compositions for inhibiting expression of a target gene in a cell, especially a mammalian cell. In certain embodiments, 15 the process comprises introduction of RNA (the "dsRNA construct") with partial or fully double-stranded character into the cell or into the extracellular environment. Inhibition is specific in that a nucleotide sequence from a portion of the target gene is chosen to produce the dsRNA construct. In preferred embodiments, the method utilizes a cell in which Dicer and/or Argonaute activities are recombinantly expressed or otherwise 20 ectopically activated. This process can be (1) effective in attenuating gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

## *II. Definitions*

25 For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to that it has been linked. One type of vector is a genomic integrated vector, or "integrated vector", which can become integrated into the 30 chromosomal DNA of the host cell. Another type of vector is an episomal vector, i.e., a nucleic acid capable of extra-chromosomal replication. Vectors capable of directing the expression of genes to that they are operatively linked are referred to herein as "expression vectors". In the present specification, "plasmid" and "vector" are used interchangeably unless otherwise clear from the context.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides.

5 As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding a polypeptide of the present invention, including both exon and (optionally) intron sequences. A "recombinant gene" refers to nucleic acid encoding such regulatory polypeptides, that may optionally include intron sequences that are derived from chromosomal DNA. The term "intron" refers to a DNA  
10 sequence present in a given gene that is not translated into protein and is generally found between exons. As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer.

15 A "protein coding sequence" or a sequence that "encodes" a particular polypeptide or peptide, is a nucleic acid sequence that is transcribed (in the case of DNA) and is translated (in the case of mRNA) into a polypeptide in vitro or in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA  
20 from prokaryotic or eukaryotic mRNA, genomic DNA sequences from prokaryotic or eukaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

25 Likewise, "encodes", unless evident from its context, will be meant to include DNA sequences that encode a polypeptide, as the term is typically used, as well as DNA sequences that are transcribed into inhibitory antisense molecules.

The term "loss-of-function", as it refers to genes inhibited by the subject RNAi method, refers a diminishment in the level of expression of a gene when compared to the level in the absence of dsRNA constructs.

30 The term "expression" with respect to a gene sequence refers to transcription of the gene and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of a protein coding sequence results from transcription and translation of the coding sequence.

35 "Cells," "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur

in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

5 By "recombinant virus" is meant a virus that has been genetically altered, e.g., by the addition or insertion of a heterologous nucleic acid construct into the particle.

As used herein, the terms "transduction" and "transfection" are art recognized and mean the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA 10 or RNA, and, for example, the transformed cell expresses a dsRNA construct.

"Transient transfection" refers to cases where exogenous DNA does not integrate into the genome of a transfected cell, e.g., where episomal DNA is transcribed into mRNA and translated into protein.

15 A cell has been "stably transfected" with a nucleic acid construct when the nucleic acid construct is capable of being inherited by daughter cells.

As used herein, a "reporter gene construct" is a nucleic acid that includes a "reporter gene" operatively linked to at least one transcriptional regulatory sequence. Transcription of the reporter gene is controlled by these sequences to which they are linked. The activity of at least one or more of these control sequences can be directly or 20 indirectly regulated by the target receptor protein. Exemplary transcriptional control sequences are promoter sequences. A reporter gene is meant to include a promoter-reporter gene construct that is heterologously expressed in a cell.

As used herein, "transformed cells" refers to cells that have spontaneously converted to a state of unrestrained growth, i.e., they have acquired the ability to grow 25 through an indefinite number of divisions in culture. Transformed cells may be characterized by such terms as neoplastic, anaplastic and/or hyperplastic, with respect to their loss of growth control. For purposes of this invention, the terms "transformed phenotype of malignant mammalian cells" and "transformed phenotype" are intended to encompass, but not be limited to, any of the following phenotypic traits associated with 30 cellular transformation of mammalian cells: immortalization, morphological or growth transformation, and tumorigenicity, as detected by prolonged growth in cell culture, growth in semi-solid media, or tumorigenic growth in immuno-incompetent or syngeneic animals.

As used herein, "proliferating" and "proliferation" refer to cells undergoing 35 mitosis.

As used herein, "immortalized cells" refers to cells that have been altered via chemical, genetic, and/or recombinant means such that the cells have the ability to grow through an indefinite number of divisions in culture.

The "growth state" of a cell refers to the rate of proliferation of the cell and the  
5 state of differentiation of the cell.

### *III. Exemplary embodiments of Isolation Method*

One aspect of the invention provides a method for potentiating RNAi by induction or ectopic activation of an RNAi enzyme in a cell (*in vivo* or *in vitro*) or cell-free mixtures. In preferred embodiments, the RNAi activity is activated or added to a mammalian cell, e.g., a human cell, which cell may be provided *in vitro* or as part of a whole organism. In other embodiments, the subject method is carried out using eukaryotic cells generally (except for oocytes) in culture. For instance, the Dicer enzyme may be activated by virtue of being recombinantly expressed or it may be activated by use of an agent which (i) induces expression of the endogenous gene, (ii) stabilizes the protein from degradation, and/or (iii) allosterically modies the enzyme to increase its activity (by altering its Kcat, Km or both).

#### A. Dicer and Argonaut Activities

20 In certain embodiment, at least one of the activated RNAi enzymes is Dicer, or a homolog thereof. In certain preferred embodiments, the present method provides for ectopic activation of Dicer. As used herein, the term "Dicer" refers to a protein which (a) mediates an RNAi response and (b) has an amino acid sequence at least 50 percent identical, and more preferably at least 75, 85, 90 or 95 percent identical to SEQ ID No. 2 or 4, and/or which can be encoded by a nucleic acid which hybridizes under wash conditions of 2 x SSC at 22°C, and more preferably 0.2 x SSC at 65°C, to a nucleotide represented by SEQ ID No. 1 or 3. Accordingly, the method may comprise introducing a dsRNA construct into a cell in which Dicer has been recombinantly expressed or otherwise ectopically activated.

30 In certain embodiment, at least one of the activated RNAi enzymes is Argonaut, or a homolog thereof. In certain preferred embodiments, the present method provides for ectopic activation of Argonaut. As used herein, the term "Argonaut" refers to a protein which (a) mediates an RNAi response and (b) has an amino acid sequence at least 50 percent identical, and more preferably at least 75, 85, 90 or 95 percent identical to the amino acid sequence shown in Figure 24. Accordingly, the method may comprise

introducing a dsRNA construct into a cell in which Argonaut has been recombinantly expressed or otherwise ectopically activated.

This invention also provides expression vectors containing a nucleic acid encoding a Dicer or Argonaut polypeptides, operably linked to at least one transcriptional regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of the subject Dicer or Argonaut proteins. Accordingly, the term transcriptional regulatory sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences, sequences that control the expression of a DNA sequence when operatively linked to it, may be used in these vectors to express DNA sequences encoding Dicer or Argonaut polypeptides of this invention. Such useful expression control sequences, include, for example, a viral LTR, such as the LTR of the Moloney murine leukemia virus, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage  $\lambda$ , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast  $\alpha$ -mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed.

Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

The recombinant Dicer or Argonaut genes can be produced by ligating nucleic acid encoding a Dicer or Argonaut polypeptide into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Expression vectors for production of recombinant forms of the subject Dicer or Argonaut polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of a Dicer or Argonaut polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived

plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach et al. (1983) in *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used. In an illustrative embodiment, a Dicer or Argonaut polypeptide is produced recombinantly utilizing an expression vector generated by sub-cloning the coding sequence of a Dicer or Argonaut gene.

The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

In yet another embodiment, the subject invention provides a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcriptional regulatory sequences of an endogenous Dicer or Argonaut gene. For instance, the gene activation construct can replace the endogenous promoter of a Dicer or Argonaut gene with a heterologous promoter, e.g., one which causes constitutive expression of the Dicer or Argonaut gene or which causes inducible expression of the gene under conditions different from the normal expression pattern of Dicer or Argonaut. A variety of different formats for the gene activation constructs are available. See, for

example, the Transkaryotic Therapies, Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

In preferred embodiments, the nucleotide sequence used as the gene activation construct can be comprised of (1) DNA from some portion of the endogenous Dicer or 5 Argonaut gene (exon sequence, intron sequence, promoter sequences, etc.) which direct recombination and (2) heterologous transcriptional regulatory sequence(s) which is to be operably linked to the coding sequence for the genomic Dicer or Argonaut gene upon recombination of the gene activation construct. For use in generating cultures of Dicer or Argonaut producing cells, the construct may further include a reporter gene to detect the 10 presence of the knockout construct in the cell.

The gene activation construct is inserted into a cell, and integrates with the genomic DNA of the cell in such a position so as to provide the heterologous regulatory sequences in operative association with the native Dicer or Argonaut gene. Such insertion occurs by homologous recombination, i.e., recombination regions of the activation 15 construct that are homologous to the endogenous Dicer or Argonaut gene sequence hybridize to the genomic DNA and recombine with the genomic sequences so that the construct is incorporated into the corresponding position of the genomic DNA.

The terms "recombination region" or "targeting sequence" refer to a segment (i.e., a portion) of a gene activation construct having a sequence that is substantially identical to 20 or substantially complementary to a genomic gene sequence, e.g., including 5' flanking sequences of the genomic gene, and can facilitate homologous recombination between the genomic sequence and the targeting transgene construct.

As used herein, the term "replacement region" refers to a portion of a activation construct which becomes integrated into an endogenous chromosomal location following 25 homologous recombination between a recombination region and a genomic sequence.

The heterologous regulatory sequences, e.g., which are provided in the replacement region, can include one or more of a variety elements, including: promoters (such as constitutive or inducible promoters), enhancers, negative regulatory elements, locus control regions, transcription factor binding sites, or combinations thereof.

Promoters/enhancers which may be used to control the expression of the targeted 30 gene *in vivo* include, but are not limited to, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al., 1989, *J. Exp. Med.*, 169:13), the human  $\beta$ -actin promoter (Gunning et al. (1987) *PNAS* 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) 35 (Klessig et al. (1984) *Mol. Cell Biol.* 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) *RNA Tumor Viruses*,

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early or late region promoter (Bernoist et al. (1981) *Nature* 290:304-310; Templeton et al. (1984) *Mol. Cell Biol.*, 4:817; and Sprague et al. (1983) *J. Virol.*, 45:773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, *Cell*, 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al. (1981) *PNAS* 82:3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) *Nature Genetics*, 1:379-384).

In still other embodiments, the replacement region merely deletes a negative transcriptional control element of the native gene, e.g., to activate expression, or ablates a positive control element, e.g., to inhibit expression of the targeted gene.

#### **B. Cell/Organism**

The cell with the target gene may be derived from or contained in any organism (e.g., plant, animal, protozoan, virus, bacterium, or fungus). The dsRNA construct may be synthesized either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or *in vitro*. For generating double stranded transcripts from a transgene *in vivo*, a regulatory region may be used to transcribe the RNA strand (or strands).

Furthermore, genetic manipulation becomes possible in organisms that are not classical genetic models. Breeding and screening programs may be accelerated by the ability to rapidly assay the consequences of a specific, targeted gene disruption. Gene disruptions may be used to discover the function of the target gene, to produce disease models in which the target gene are involved in causing or preventing a pathological condition, and to produce organisms with improved economic properties.

The cell with the target gene may be derived from or contained in any organism. The organism may a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies.

Plants include arabidopsis; field crops (e.g., alfalfa, barley, bean, corn, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, and wheat); vegetable crops (e.g., asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, 35 tomato, and zucchini); fruit and nut crops (e.g., almond, apple, apricot, banana, blackberry,

blueberry, cacao, cherry, coconut, cranberry, date, fajoa, filbert, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon); and ornamentals (e.g., alder, ash, aspen, azalea, birch, boxwood, camellia, 5 carnation, chrysanthemum, elm, fir, ivy, jasmine, juniper, oak, palm, poplar, pine, redwood, rhododendron, rose, and rubber).

Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human.

Invertebrate animals include nematodes, other worms, drosophila, and other 10 insects. Representative genera of nematodes include those that infect animals (e.g., Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haernonchus, Heterakis, Nematodirus, Oesophagostomum, Ostertagia, Oxyuris, Parascaris, Strongylus, Toxascaris, Trichuris, Trichostrongylus, Tflichonema, Toxocara, Uncinaria) and those that infect plants (e.g., B ursaphalenchus, 15 Criconerriella, Diylenchus, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Longidorus, Melodoigyne, Nacobbus, Paratylenchus, Pratylenchus, Radopholus, Rotelychnus, Tylenchus, and Xiphinema). Representative orders of insects include Coleoptera, Diptera, Lepidoptera, and Homoptera.

The cell having the target gene may be from the germ line or somatic, totipotent or 20 pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, 25 osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

### C. Targeted Genes

The target gene may be a gene derived from the cell, an endogenous gene, a transgene, or a gene of a pathogen which is present in the cell after infection thereof. 30 Depending on the particular target gene and the dose of double stranded RNA material delivered, the procedure may provide partial or complete loss of function for the target gene. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells. Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA 35 or translation of target protein.

“Inhibition of gene expression” refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene. “Specificity” refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The consequences of inhibition can be confirmed by examination of the outward properties of 5 the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radiolmmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). For RNA-mediated 10 inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxyacid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucuronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), 15 nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracycline.

Depending on the assay, quantitation of the amount of gene expression allows one 20 to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells (e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted 25 cells). Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein. As an example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell: mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

30 As disclosed herein, the present invention may not be limited to any type of target gene or nucleotide sequence. But the following classes of possible target genes are listed for illustrative purposes: developmental genes (e.g., adhesion molecules, cyclin kinase inhibitors, Writ family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation 35 factors and their receptors, neurotransmitters and their receptors); oncogenes (e.g., ABL1, BCLI, BCL2, BCL6, CBFA2, CBL, CSF1R, ERBA, ERBB, EBRB2, ETS1, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC,

MYCLI, MYCN, NRAS, PIM 1, PML, RET, SRC, TALI, TCL3, and YES); tumor suppressor genes (e.g., APC, BRCA 1, BRCA2, MADH4, MCC, NF 1, NF2, RB 1, TP53, and WT1); and enzymes (e.g., ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases, 5 amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerasases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, 10 phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerase, and xylanases).

#### D. dsRNA constructs

15 The dsRNA construct may comprise one or more strands of polymerized ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored to allow specific genetic inhibition while avoiding a general panic 20 response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. The dsRNA construct may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by in vitro enzymatic or organic synthesis.

25 The dsRNA construct may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing RNA. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the organism to be affected. Physical methods 30 of introducing nucleic acids include injection directly into the cell or extracellular injection into the organism of an RNA solution.

35 The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded material may yield more effective inhibition; lower

doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

dsRNA constructs containing a nucleotide sequences identical to a portion of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C hybridization for 12-16 hours; followed by washing). The length of the identical nucleotide sequences may be, for example, at least 25, 50, 100, 200, 300 or 400 bases. In certain embodiments, the dsRNA construct is 400-800 bases in length.

100% sequence identity between the RNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

The dsRNA construct may be synthesized either in vivo or in vitro. Endogenous RNA polymerase of the cell may mediate transcription in vivo, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For transcription from a transgene in vivo or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to transcribe the dsRNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; stimulation of an environmental condition (e.g., infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. The dsRNA construct may be chemically or enzymatically synthesized by manual or automated reactions. The dsRNA construct may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and

production of an expression construct are known in the art<sup>32,33,34</sup> (see also WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by in vitro enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be 5 purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography or a combination thereof. Alternatively, the dsRNA construct may be used with no or a minimum of purification to avoid losses due to sample processing.. The dsRNA construct may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or 10 stabilization of the duplex strands.

Physical methods of introducing nucleic acids include injection of a solution containing the dsRNA construct, bombardment by particles covered by the dsRNA construct, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the dsRNA construct. A viral construct packaged into a viral 15 particle would accomplish both efficient introduction of an expression construct into the cell and transcription of dsRNA construct encoded by the expression construct. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemicalmediated transport, such as calcium phosphate, and the like. Thus the dsRNA construct may be introduced along with components that 20 perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or other-wise increase inhibition of the target gene.

#### E. Illustrative Uses

25 One utility of the present invention is as a method of identifying gene function in an organism, especially higher eukaryotes comprising the use of double-stranded RNA to inhibit the activity of a target gene of previously unknown function. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics would envision determining the function of uncharacterized genes by employing 30 the invention to reduce the amount and/or alter the timing of target gene activity. The invention could be used in determining potential targets for pharmaceutics, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, 35 including total sequences for mammalian genomes, can be coupled with the invention to determine gene function in a cell or in a whole organism. The preference of different

organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such 5 sequencing projects.

A simple assay would be to inhibit gene expression according to the partial sequence available from an expressed sequence tag (EST). Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

10 The ease with which the dsRNA construct can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, duplex RNA can be produced by an amplification reaction using primers flanking the inserts of any gene library derived from the target cell/organism. Inserts may be derived from genomic DNA or mRNA (e.g., 15 cDNA and cRNA). Individual clones from the library can be replicated and then isolated in separate reactions, but preferably the library is maintained in individual reaction vessels (e.g., a 96 well microtiter plate) to minimize the number of steps required to practice the invention and to allow automation of the process. Solutions containing duplex RNAs that are capable of inhibiting the different expressed genes can be placed into individual wells 20 positioned on a microtiter plate as an ordered array, and intact cells/organisms in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity. The amplified RNA can be fed directly to, injected into, the cell/organism containing the target gene. Alternatively, the duplex RNA can be produced by in vivo or in vitro transcription from an expression construct used to produce 25 the library. The construct can be replicated as individual clones of the library and transcribed to produce the RNA; each clone can then be fed to, or injected into, the cell/organism containing the target gene. The function of the target gene can be assayed from the effects it has on the cell/organism when gene activity is inhibited. This screening could be amenable to small subjects that can be processed in large number, for example, 30 tissue culture cells derived from mammals, especially primates, and most preferably humans.

If a characteristic of an organism is determined to be genetically linked to a polymorphism through RFLP or QTL analysis, the present invention can be used to gain insight regarding whether that genetic polymorphism might be directly responsible for the 35 characteristic. For example, a fragment defining the genetic polymorphism or sequences in the vicinity of such a genetic polymorphism can be amplified to produce an RNA, the

duplex RNA can be introduced to the organism or cell, and whether an alteration in the characteristic is correlated with inhibition can be determined. Of course, there may be trivial explanations for negative results with this type of assay, for example: inhibition of the target gene causes lethality, inhibition of the target gene may not result in any 5 observable alteration, the fragment contains nucleotide sequences that are not capable of inhibiting the target gene, or the target gene's activity is redundant.

The present invention may be useful in allowing the inhibition of essential genes. Such genes may be required for cell or organism viability at only particular stages of development or cellular compartments. The functional equivalent of conditional mutations 10 may be produced by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of RNA at specific times of development and locations in the organism without introducing permanent mutations into the target genome.

If alternative splicing produced a family of transcripts that were distinguished by usage of characteristic exons, the present invention can target inhibition through the 15 appropriate exons to specifically inhibit or to distinguish among the functions of family members. For example, a hormone that contained an alternatively spliced transmembrane domain may be expressed in both membrane bound and secreted forms. Instead of isolating a nonsense mutation that terminates translation before the transmembrane domain, the functional consequences of having only secreted hormone can be determined 20 according to the invention by targeting the exon containing the transmembrane domain and thereby inhibiting expression of membrane-bound hormone.

The present invention may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the in vitro or in vivo introduction of RNA to 25 test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

Alternatively, an organism may be engineered to produce dsRNA which produces commercially or medically beneficial results, for example, resistance to a pathogen or its pathogenic effects, improved growth, or novel developmental patterns.

30

#### *IV. Exemplification*

The invention, now being generally described, will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention and are not intended to limit 35 the invention.

**Example 1: An RNA-directed nuclease mediates RNAi gene silencing**

In a diverse group of organisms that includes *Caenorhabditis elegans*, *Drosophila*, planaria, hydra, trypanosomes, fungi and plants, the introduction of double-stranded RNAs 5 inhibits gene expression in a sequence-specific manner<sup>1-7</sup>. These responses, called RNA interference or post-transcriptional gene silencing, may provide anti-viral defence, modulate transposition or regulate gene expression<sup>1, 6, 8-10</sup>. We have taken a biochemical approach towards elucidating the mechanisms underlying this genetic phenomenon. Here 10 we show that 'loss-of-function' phenotypes can be created in cultured *Drosophila* cells by transfection with specific double-stranded RNAs. This coincides with a marked reduction in the level of cognate cellular messenger RNAs. Extracts of transfected cells contain a 15 nuclease activity that specifically degrades exogenous transcripts homologous to transfected double-stranded RNA. This enzyme contains an essential RNA component. After partial purification, the sequence-specific nuclease co-fractionates with a discrete, ~25-nucleotide RNA species which may confer specificity to the enzyme through homology to the substrate mRNAs.

Although double-stranded RNAs (dsRNAs) can provoke gene silencing in numerous biological contexts including *Drosophila*<sup>11, 12</sup>, the mechanisms underlying this phenomenon have remained mostly unknown. We therefore wanted to establish a 20 biochemically tractable model in which such mechanisms could be investigated.

Transient transfection of cultured, *Drosophila* S2 cells with a *lacZ* expression vector resulted in  $\beta$ -galactosidase activity that was easily detectable by an *in situ* assay 25 (Fig. 1a). This activity was greatly reduced by co-transfection with a dsRNA corresponding to the first 300 nucleotides of the *lacZ* sequence, whereas co-transfection with a control dsRNA (CD8) (Fig. 1a) or with single-stranded RNAs of either sense or antisense orientation (data not shown) had little or no effect. This indicated that dsRNAs could interfere, in a sequence-specific fashion, with gene expression in cultured cells.

To determine whether RNA interference (RNAi) could be used to target endogenous genes, we transfected S2 cells with a dsRNA corresponding to the first 30 540 nucleotides of *Drosophila cyclin E*, a gene that is essential for progression into S phase of the cell cycle. During log-phase growth, untreated S2 cells reside primarily in G2/M (Fig. 1b). Transfection with *lacZ* dsRNA had no effect on cell-cycle distribution, but 35 transfection with the *cyclin E* dsRNA caused a G1-phase cell-cycle arrest (Fig. 1b). The ability of *cyclin E* dsRNA to provoke this response was length-dependent. Double-stranded RNAs of 540 and 400 nucleotides were quite effective, whereas dsRNAs of 200 and 300 nucleotides were less potent. Double-stranded *cyclin E* RNAs of 50 or 100

nucleotides were inert in our assay, and transfection with a single-stranded, antisense *cyclin E* RNA had virtually no effect.

One hallmark of RNAi is a reduction in the level of mRNAs that are homologous to the dsRNA. Cells transfected with the *cyclin E* dsRNA (bulk population) showed 5 diminished endogenous *cyclin E* mRNA as compared with control cells (Fig. 1c). Similarly, transfection of cells with dsRNAs homologous to *fizzy*, a component of the anaphase-promoting complex (APC) or *cyclin A*, a cyclin that acts in S, G2 and M, also caused reduction of their cognate mRNAs (Fig. 1c). The modest reduction in *fizzy* mRNA levels in cells transfected with *cyclin A* dsRNA probably resulted from arrest at a point in 10 the division cycle at which *fizzy* transcription is low<sup>14, 15</sup>. These results indicate that RNAi may be a generally applicable method for probing gene function in cultured *Drosophila* cells.

The decrease in mRNA levels observed upon transfection of specific dsRNAs into 15 *Drosophila* cells could be explained by effects at transcriptional or post-transcriptional levels. Data from other systems have indicated that some elements of the dsRNA response may affect mRNA directly (reviewed in refs 1 and 6). We therefore sought to develop a cell-free assay that reflected, at least in part, RNAi.

S2 cells were transfected with dsRNAs corresponding to either *cyclin E* or *lacZ*. Cellular extracts were incubated with synthetic mRNAs of *lacZ* or *cyclin E*. Extracts 20 prepared from cells transfected with the 540-nucleotide *cyclin E* dsRNA efficiently degraded the *cyclin E* transcript; however, the *lacZ* transcript was stable in these lysates (Fig. 2a). Conversely, lysates from cells transfected with the *lacZ* dsRNA degraded the *lacZ* transcript but left the *cyclin E* mRNA intact. These results indicate that RNAi ablates target mRNAs through the generation of a sequence-specific nuclease activity. We have 25 termed this enzyme RISC (RNA-induced silencing complex). Although we occasionally observed possible intermediates in the degradation process (see Fig. 2), the absence of stable cleavage end-products indicates an exonuclease (perhaps coupled to an endonuclease). However, it is possible that the RNAi nuclease makes an initial endonucleolytic cut and that non-specific exonucleases in the extract complete the 30 degradation process<sup>16</sup>. In addition, our ability to create an extract that targets *lacZ* *in vitro* indicates that the presence of an endogenous gene is not required for the RNAi response.

To examine the substrate requirements for the dsRNA-induced, sequence-specific nuclease activity, we incubated a variety of *cyclin-E*-derived transcripts with an extract derived from cells that had been transfected with the 540-nucleotide *cyclin E* dsRNA (Fig. 35 2b, c). Just as a length requirement was observed for the transfected dsRNA, the RNAi nuclease activity showed a dependence on the size of the RNA substrate. Both a 600-

nucleotide transcript that extends slightly beyond the targeted region (Fig. 2b) and an ~1-kilobase (kb) transcript that contains the entire coding sequence (data not shown) were completely destroyed by the extract. Surprisingly, shorter substrates were not degraded as efficiently. Reduced activity was observed against either a 300- or a 220-nucleotide 5 transcript, and a 100-nucleotide transcript was resistant to nuclease in our assay. This was not due solely to position effects because ~100-nucleotide transcripts derived from other portions of the transfected dsRNA behaved similarly (data not shown). As expected, the nuclease activity (or activities) present in the extract could also recognize the antisense strand of the *cyclin E* mRNA. Again, substrates that contained a substantial portion of the 10 targeted region were degraded efficiently whereas those that contained a shorter stretch of homologous sequence (~130 nucleotides) were recognized inefficiently (Fig. 2c, as600). For both the sense and antisense strands, transcripts that had no homology with the transfected dsRNA (Fig. 2b, Eout; Fig. 2c, as300) were not degraded. Although we cannot exclude the possibility that nuclease specificity could have migrated beyond the targeted 15 region, the resistance of transcripts that do not contain homology to the dsRNA is consistent with data from *C. elegans*. Double-stranded RNAs homologous to an upstream cistron have little or no effect on a linked downstream cistron, despite the fact that unprocessed, polycistronic mRNAs can be readily detected<sup>17, 18</sup>. Furthermore, the nuclease was inactive against a dsRNA identical to that used to provoke the RNAi response *in vivo* 20 (Fig. 2b). In the *in vitro* system, neither a 5' cap nor a poly(A) tail was required, as such transcripts were degraded as efficiently as uncapped and non-polyadenylated RNAs.

Gene silencing provoked by dsRNA is sequence specific. A plausible mechanism for determining specificity would be incorporation of nucleic-acid guide sequences into the complexes that accomplish silencing<sup>19</sup>. In accord with this idea, pre-treatment of 25 extracts with a Ca<sup>2+</sup>-dependent nuclease (micrococcal nuclease) abolished the ability of these extracts to degrade cognate mRNAs (Fig. 3). Activity could not be rescued by addition of non-specific RNAs such as yeast transfer RNA. Although micrococcal nuclease can degrade both DNA and RNA, treatment of the extract with DNase I had no effect (Fig. 3). Sequence-specific nuclease activity, however, did require protein (data not 30 shown). Together, our results support the possibility that the RNAi nuclease is a ribonucleoprotein, requiring both RNA and protein components. Biochemical fractionation (see below) is consistent with these components being associated in extract rather than being assembled on the target mRNA after its addition.

In plants, the phenomenon of co-suppression has been associated with the 35 existence of small (~25-nucleotide) RNAs that correspond to the gene that is being silenced<sup>19</sup>. To address the possibility that a similar RNA might exist in *Drosophila* and guide the sequence-specific nuclease in the choice of substrate, we partially purified our

activity through several fractionation steps. Crude extracts contained both sequence-specific nuclease activity and abundant, heterogeneous RNAs homologous to the transfected dsRNA (Figs 2 and 4a). The RNAi nuclease fractionated with ribosomes in a high-speed centrifugation step. Activity could be extracted by treatment with high salt, and 5 ribosomes could be removed by an additional centrifugation step. Chromatography of soluble nuclease over an anion-exchange column resulted in a discrete peak of activity (Fig. 4b, *cyclin E*). This retained specificity as it was inactive against a heterologous mRNA (Fig. 4b, *lacZ*). Active fractions also contained an RNA species of 25 nucleotides that is homologous to the *cyclin E* target (Fig. 4b, northern). The band observed on 10 northern blots may represent a family of discrete RNAs because it could be detected with probes specific for both the sense and antisense *cyclin E* sequences and with probes derived from distinct segments of the dsRNA (data not shown). At present, we cannot determine whether the 25-nucleotide RNA is present in the nuclease complex in a double-stranded or single-stranded form.

15 RNA interference allows an adaptive defence against both exogenous and endogenous dsRNAs, providing something akin to a dsRNA immune response. Our data, and that of others<sup>19</sup>, is consistent with a model in which dsRNAs present in a cell are converted, either through processing or replication, into small specificity determinants of discrete size in a manner analogous to antigen processing. Our results suggest that the 20 post-transcriptional component of dsRNA-dependent gene silencing is accomplished by a sequence-specific nuclease that incorporates these small RNAs as guides that target specific messages based upon sequence recognition. The identical size of putative specificity determinants in plants<sup>19</sup> and animals predicts a conservation of both the mechanisms and the components of dsRNA-induced, post-transcriptional gene silencing in 25 diverse organisms. In plants, dsRNAs provoke not only post-transcriptional gene silencing but also chromatin remodelling and transcriptional repression<sup>20, 21</sup>. It is now critical to determine whether conservation of gene-silencing mechanisms also exists at the transcriptional level and whether chromatin remodelling can be directed in a sequence-specific fashion by these same dsRNA-derived guide sequences.

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### Methods

Cell culture and RNA methods S2 (ref. 22) cells were cultured at 27 °C in 90% Schneider's insect media (Sigma), 10% heat inactivated fetal bovine serum (FBS). Cells were transfected with dsRNA and plasmid DNA by calcium phosphate co-precipitation<sup>23</sup>. 35 Identical results were observed when cells were transfected using lipid reagents (for example, Superfect, Qiagen). For FACS analysis, cells were additionally transfected with

a vector that directs expression of a green fluorescent protein (GFP)–US9 fusion protein<sup>13</sup>. These cells were fixed in 90% ice-cold ethanol and stained with propidium iodide at 25 µg ml<sup>-1</sup>. FACS was performed on an Elite flow cytometer (Coulter). For northern blotting, equal loading was ensured by over-probing blots with a control complementary DNA (RP49). For the production of dsRNA, transcription templates were generated by polymerase chain reaction such that they contained T7 promoter sequences on each end of the template. RNA was prepared using the RiboMax kit (Promega). Confirmation that RNAs were double stranded came from their complete sensitivity to RNase III (a gift from A. Nicholson). Target mRNA transcripts were synthesized using the Riboprobe kit (Promega) and were gel purified before use.

**Extract preparation** Log-phase S2 cells were plated on 15-cm tissue culture dishes and transfected with 30 µg dsRNA and 30 µg carrier plasmid DNA. Seventy-two hours after transfection, cells were harvested in PBS containing 5 mM EGTA washed twice in PBS and once in hypotonic buffer (10 mM HEPES pH 7.3, 6 mM β-mercaptoethanol). Cells were suspended in 0.7 packed-cell volumes of hypotonic buffer containing *Complete* protease inhibitors (Boehringer) and 0.5 units ml<sup>-1</sup> of RNasin (Promega). Cells were disrupted in a dounce homogenizer with a type B pestle, and lysates were centrifuged at 30,000g for 20 min. Supernatants were used in an *in vitro* assay containing 20 mM HEPES pH 7.3, 110 mM KOAc, 1 mM Mg(OAc)<sub>2</sub>, 3 mM EGTA, 2 mM CaCl<sub>2</sub>, 1 mM DTT. Typically, 5 µl extract was used in a 10 µl assay that contained also 10,000 c.p.m. synthetic mRNA substrate.

**Extract fractionation** Extracts were centrifuged at 200,000g for 3 h and the resulting pellet (containing ribosomes) was extracted in hypotonic buffer containing also 1 mM MgCl<sub>2</sub> and 300 mM KOAc. The extracted material was spun at 100,000g for 1 h and the resulting supernatant was fractionated on Source 15Q column (Pharmacia) using a KCl gradient in buffer A (20 mM HEPES pH 7.0, 1 mM dithiothreitol, 1 mM MgCl<sub>2</sub>). Fractions were assayed for nuclease activity as described above. For northern blotting, fractions were proteinase K/SDS treated, phenol extracted, and resolved on 15% acrylamide 8M urea gels. RNA was electroblotted onto Hybond N+ and probed with strand-specific riboprobes derived from cyclin E mRNA. Hybridization was carried out in 500 mM NaPO<sub>4</sub> pH 7.0, 15% formamide, 7% SDS, 1% BSA. Blots were washed in 1 SSC at 37–45 °C.

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**Example 2: Role for a bidentate ribonuclease in the initiation step of RNA interference**

25 Genetic approaches in worms, fungi and plants have identified a group of proteins that are essential for double-stranded RNA-induced gene silencing. Among these are ARGONAUTE family members (e.g. RDE1, QDE2)<sup>9,10,30</sup>, recQ-family helicases (MUT-7, QDE3)<sup>11,12</sup>, and RNA-dependent RNA polymerases (e.g. EGO-1, QDE1, SGS2/SDE1)<sup>13-16</sup>. While potential roles have been proposed, none of these genes has been assigned a definitive function in the silencing process. Biochemical studies have suggested that 30 PTGS is accomplished by a multicomponent nuclease that targets mRNAs for degradation<sup>6,8,17</sup>. We have shown that the specificity of this complex may derive from the incorporation of a small guide sequence that is homologous to the mRNA substrate<sup>6</sup>. Originally identified in plants that were actively silencing transgenes<sup>7</sup>, these ~22 nt. RNAs

have been produced during RNAi *in vitro* using an extract prepared from *Drosophila* embryos<sup>8</sup>. Putative guide RNAs can also be produced in extracts from *Drosophila* S2 cells (Fig. 5a). With the goal of understanding the mechanism of post-transcriptional gene silencing, we have undertaken both biochemical fractionation and candidate gene 5 approaches to identify the enzymes that execute each step of RNAi.

Our previous studies resulted in the partial purification of a nuclease, RISC, that is an effector of RNA interference. See Example 1. This enzyme was isolated from *Drosophila* S2 cells in which RNAi had been initiated *in vivo* by transfection with dsRNA. We first sought to determine whether the RISC enzyme and the enzyme that initiates 10 RNAi via processing of dsRNA into 22mers are distinct activities. RISC activity could be largely cleared from extracts by high-speed centrifugation (100,000xg for 60 min.) while the activity that produces 22mers remained in the supernatant (Fig. 5b,c). This simple fractionation indicated that RISC and the 22mer-generating activity are separable and thus 15 distinct enzymes. However, it seems likely that they might interact at some point during the silencing process.

RNAse III family members are among the few nucleases that show specificity for double-stranded RNA<sup>18</sup>. Analysis of the *Drosophila* and *C. elegans* genomes reveals several types of RNAse III enzymes. First is the canonical RNAse III which contains a single RNAse III signature motif and a double-stranded RNA binding domain (dsRBD; 20 e.g. RNC\_CAEEL). Second is a class represented by Drosha<sup>19</sup>, a *Drosophila* enzyme that contains two RNAse III motifs and a dsRBD (CeDrosha in *C. elegans*). A third class contains two RNAse III signatures and an amino terminal helicase domain (e.g. *Drosophila* CG4792, CG6493, *C. elegans* K12H4.8), and these had previously been proposed by Bass as candidate RNAi nucleases<sup>20</sup>. Representatives of all three classes 25 were tested for the ability to produce discrete, ~22 nt. RNAs from dsRNA substrates.

Partial digestion of a 500 nt. cyclin E dsRNA with purified, bacterial RNAse III produced a smear of products while nearly complete digestion produced a heterogeneous group of ~11-17 nucleotide RNAs (not shown). In order to test the dual-RNAse III enzymes, we prepared T7 epitope-tagged versions of Drosha and CG4792. These were 30 expressed in transfected S2 cells and isolated by immunoprecipitation using antibody-agarose conjugates. Treatment of the dsRNA with the CG4792 immunoprecipitate yielded ~22 nt. fragments similar to those produced in either S2 or embryo extracts (Fig. 6a). Neither activity in extract nor activity in immunoprecipitates depended on the sequence of the RNA substrate since dsRNAs derived from several genes were processed equivalently 35 (see Supplement 1). Negative results were obtained with Drosha and with immunoprecipitates of a DExH box helicase (Homeless<sup>21</sup>; see Fig 6a,b). Western blotting

confirmed that each of the tagged proteins was expressed and immunoprecipitated similarly (see Supplement 2). Thus, we conclude that CG4792 may carry out the initiation step of RNA interference by producing ~22 nt. guide sequences from dsRNAs. Because of its ability to digest dsRNA into uniformly sized, small RNAs, we have named this 5 enzyme Dicer (*Dcr*). *Dicer* mRNA is expressed in embryos, in S2 cells, and in adult flies, consistent with the presence of functional RNAi machinery in all of these contexts (see Supplement 3).

The possibility that Dicer might be the nuclease responsible for the production of 10 guide RNAs from dsRNAs prompted us to raise an antiserum directed against the carboxy- terminus of the Dicer protein (Dicer-1, CG4792). This antiserum could immunoprecipitate a nuclease activity from either Drosophila embryo extracts or from S2 cell lysates that produced ~22 nt. RNAs from dsRNA substrates (Fig. 6C). The putative 15 guide RNAs that are produced by the Dicer-1 enzyme precisely comigrate with 22mers that are produced in extract and with 22mers that are associated with the RISC enzyme (Fig. 6 D,F). It had previously been shown that the enzyme that produced guide RNAs in Drosophila embryo extracts was ATP-dependent<sup>8</sup>. Depletion of this cofactor resulted in an ~6-fold lower rate of dsRNA cleavage and in the production of RNAs with a slightly 20 lower mobility. Of interest was the fact that both Dicer-1 immunoprecipitates and extracts from S2 cells require ATP for the production of ~22mers (Fig. 6D). We do not observe the accumulation of lower mobility products in these cases, although we do routinely 25 observe these in ATP-depleted embryo extracts. The requirement of this nuclease for ATP is a quite unusual property. We hypothesize that this requirement could indicate that the enzyme may act processively on the dsRNA, with the helicase domain harnessing the energy of ATP hydrolysis both for unwinding guide RNAs and for translocation along the substrate.

Efficient induction of RNA interference in *C. elegans* and in *Drosophila* has 30 several requirements. For example, the initiating RNA must be double-stranded, and it must be several hundred nucleotides in length. To determine whether these requirements are dictated by Dicer, we characterized the ability of extracts and of immunoprecipitated 35 enzyme to digest various RNA substrates. Dicer was inactive against single stranded RNAs regardless of length (see Supplement 4). The enzyme could digest both 200 and 500 nucleotide dsRNAs but was significantly less active with shorter substrates (see Supplement 4). Double-stranded RNAs as short as 35 nucleotides could be cut by the enzyme, albeit very inefficiently (data not shown). In contrast, *E. coli* RNase III could digest to completion dsRNAs of 35 or 22 nucleotides (not shown). This suggests that the 35 substrate preferences of the Dicer enzyme may contribute to but not wholly determine the size dependence of RNAi.

To determine whether the Dicer enzyme indeed played a role in RNAi *in vivo*, we sought to deplete Dicer activity from S2 cells and test the effect on dsRNA-induced gene silencing. Transfection of S2 cells with a mixture of dsRNAs homologous to the two Drosophila Dicer genes (CG4792 and CG6493) resulted in an ~6-7 fold reduction of Dicer 5 activity either in whole cell lysates or in Dicer-1 immunoprecipitates (Fig. 7A,B). Transfection with a control dsRNA (murine caspase 9) had no effect. Qualitatively similar results were seen if Dicer was examined by Northern blotting (not shown). Depletion of Dicer in this manner substantially compromised the ability of cells to silence subsequently an exogenous, GFP transgene by RNAi (Fig. 7C). These results indicate that Dicer is 10 involved in RNAi *in vivo*. The lack of complete inhibition of silencing could result from an incomplete suppression of Dicer (which is itself required for RNAi) or could indicate that *in vivo*, guide RNAs can be produced by more than one mechanism (e.g. through the action of RNA-dependent RNA polymerases).

Our results indicate that the process of RNA interference can be divided into at 15 least two distinct steps. According to this model, initiation of PTGS would occur upon processing of a double-stranded RNA by Dicer into ~22 nucleotide guide sequences, although we cannot formally exclude the possibility that another, Dicer-associated nuclease may participate in this process. These guide RNAs would be incorporated into a distinct nuclease complex (RISC) that targets single-stranded mRNAs for degradation. An 20 implication of this model is that guide sequences are themselves derived directly from the dsRNA that triggers the response. In accord with this model, we have demonstrated that <sup>32</sup>P-labeled, exogenous dsRNAs that have been introduced into S2 cells by transfection are incorporated into the RISC enzyme as 22 mers (Fig. 7E). However, we cannot exclude the possibility that RNA-dependent RNA polymerases might amplify 22mers once they have 25 been generated or provide an alternative method for producing guide RNAs.

The structure of the Dicer enzyme provokes speculation on the mechanism by which the enzyme might produce discretely sized fragments irrespective of the sequence of the dsRNA (see Supplement 1, Fig. 8a). It has been established that bacterial RNase III acts on its substrate as a dimer<sup>18,22,23</sup>. Similarly, a dimer of Dicer enzymes may be 30 required for cleavage of dsRNAs into ~22 nt. pieces. According to one model, the cleavage interval would be determined by the physical arrangement of the two RNase III domains within Dicer enzyme (Fig. 8a). A plausible alternative model would dictate that cleavage was directed at a single position by the two RIII domains in a single Dicer 35 enzymes or by translocation along the mRNA substrate. The presence of an integral helicase domain suggests that the products of Dicer cleavage might be single-stranded 22 mers that are incorporated into the RISC enzyme as such.

A notable feature of the Dicer family is its evolutionary conservation. Homologs are found in *C. elegans* (K12H4.8), *Arabidopsis* (e.g., CARPEL FACTORY<sup>24</sup>, T25K16.4, AC012328\_1), mammals (Helicase-MOI<sup>25</sup>) and *S. pombe* (YC9A\_SCHPO) (Fig 8b, see Supplements 6,7 for sequence comparisons). In fact, the human Dicer family member is 5 capable of generating ~22 nt. RNAs from dsRNA substrates (Supplement 5) suggesting that these structurally similar proteins may all share similar biochemical functions. It has been demonstrated that exogenous dsRNAs can affect gene function in early mouse embryos<sup>29</sup>, and our results suggest that this regulation may be accomplished by an evolutionarily conserved RNAi machinery.

10 In addition to RNaseIII and helicase motifs, searches of the PFAM database indicate that each Dicer family member also contains a ZAP domain (Fig 8c)<sup>27</sup>. This sequence was defined based solely upon its conservation in the Zwille/ARGONAUTE/Piwi family that has been implicated in RNAi by mutations in *C. elegans* (Rde-1)<sup>9</sup> and *Neurospora* (Qde-2)<sup>10</sup>. Although the function of this domain is 15 unknown, it is intriguing that this region of homology is restricted to two gene families that participate in dsRNA-dependent silencing. Both the ARGONAUTE and Dicer families have also been implicated in common biological processes, namely the determination of stem-cell fates. A hypomorphic allele of *carpel factory*, a member of the Dicer family in *Arabidopsis*, is characterized by increased proliferation in floral 20 meristems<sup>24</sup>. This phenotype and a number of other characteristic features are also shared by *Arabidopsis ARGONAUTE (ago1-1)* mutants<sup>26</sup> (C. Kidner and R. Martienssen, pers. comm.). These genetic analyses begin to provide evidence that RNAi may be more than a defensive response to unusual RNAs but may also play important roles in the regulation of endogenous genes.

25 With the identification of Dicer as a catalyst of the initiation step of RNAi, we have begun to unravel the biochemical basis of this unusual mechanism of gene regulation. It will be of critical importance to determine whether the conserved family members from other organisms, particularly mammals, also play a role in dsRNA-mediated gene regulation.

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### Methods

Plasmid constructs. A full-length cDNA encoding Drosha was obtained by PCR from an EST sequenced by the Berkeley Drosophila genome project. The *Homeless* clone was a gift from Gillespie and Berg (Univ. Washington). The T7 epitope-tag was added to 35 the amino terminus of each by PCR, and the tagged cDNAs were cloned into pRIP, a retroviral vector designed specifically for expression in insect cells (E. Bernstein,

unpublished). In this vector, expression is driven by the *Orgyia pseudotsugata* IE2 promoter (Invitrogen). Since no cDNA was available for CG4792/Dicer, a genomic clone was amplified from a bacmid (BACR23F10; obtained from the BACPAC Resource Center in the Dept. of Human Genetics at the Roswell Park Cancer Institute). Again, during 5 amplification, a T7 epitope tag was added at the amino terminus of the coding sequence. The human Dicer gene was isolated from a cDNA library prepared from HaCaT cells (GJH, unpublished). A T7-tagged version of the complete coding sequence was cloned into pCDNA3 (Invitrogen) for expression in human cells (LinX-A).

Cell culture and extract preparation. *S2 and embryo culture.* S2 cells were 10 cultured at 27°C in 5% CO<sub>2</sub> in Schneider's insect media supplemented with 10% heat inactivated fetal bovine serum (Gemini) and 1% antibiotic-antimycotic solution (Gibco BRL). Cells were harvested for extract preparation at 10x10<sup>6</sup> cells/ml. The cells were washed 1X in PBS and were resuspended in a hypotonic buffer (10 mM Hepes pH 7.0, 2mM MgCl<sub>2</sub>, 6 mM βME) and dounced. Cell lysates were spun 20,000xg for 20 minutes. 15 Extracts were stored at -80°C. *Drosophila* embryos were reared in fly cages by standard methodologies and were collected every 12 hours. The embryos were dechorionated in 50% chlorox bleach and washed thoroughly with distilled water. Lysis buffer (10mM Hepes, 10mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.5mM EGTA, 10mM β-glycerophosphate, 1mM DTT, 0.2 mM PMSF) was added to the embryos, and extracts were prepared by 20 homogenization in a tissue grinder. Lysates were spun for two hours at 200,000xg and were frozen at -80°C. LinX-A cells, a highly-transflectable derivative of human 293 cells, (Lin Xie and GJH, unpublished) were maintained in DMEM/10%FCS.

Transfections and immunoprecipitations. S2 cells were transfected using a calcium phosphate procedure essentially as previously described<sup>6</sup>. Transfection rates were ~90% 25 as monitored in controls using an *in situ* β-galactosidase assay. LinX-A cells were also transfected by calcium phosphate co-precipitation. For immunoprecipitations, cells (~ 5x10<sup>6</sup> per IP) were transfected with various clones and lysed three days later in IP buffer (125mM KOAc, 1mM MgOAc, 1mM CaCl<sub>2</sub>, 5mM EGTA, 20mM Hepes pH 7.0, 1mM DTT, 1% NP-40 plus Complete protease inhibitors (Roche)). Lysates were spun for 10 30 minutes at 14,000xg and supernatants were added to T7 antibody-agarose beads (Novagen). Antibody binding proceeded for 4 hours at 4°C. Beads were centrifuged and washed in lysis buffer three times, and once in reaction buffer. The Dicer antiserum was raised in rabbits using a KLH-conjugated peptide corresponding to the C-terminal 8 amino acids of *Drosophila* Dicer-1 (CG4792).

35 Cleavage reactions. *RNA preparation.* Templates to be transcribed into dsRNA were generated by PCR with forward and reverse primers, each containing a T7 promoter

sequence. RNAs were produced using Riboprobe (Promega) kits and were uniformly labeling during the transcription reaction with  $^{32}\text{P}$ -UTP. Single-stranded RNAs were purified from 1% agarose gels. *dsRNA cleavage*. Five microliters of embryo or S2 extracts were incubated for one hour at 30°C with dsRNA in a reaction containing 20mM 5 Hepes pH 7.0, 2mM MgOAc, 2mM DTT, 1mM ATP and 5% Superasin (Ambion). Immunoprecipitates were treated similarly except that a minimal volume of reaction buffer (including ATP and Superasin) and dsRNA were added to beads that had been washed in reaction buffer (see above). For ATP depletion, *Drosophila* embryo extracts were incubated for 20 minutes at 30°C with 2mM glucose and 0.375 U of hexokinase (Roche) 10 prior to the addition of dsRNA.

*Northern and Western analysis*. Total RNA was prepared from *Drosophila* embryos (0-12 hour), from adult flies, and from S2 cells using Trizol (Lifetech). Messenger RNA was isolated by affinity selection using magnetic oligo-dT beads (Dynal). RNAs were electrophoresed on denaturing formaldehyde/agarose gels, blotted and probed 15 with randomly primed DNAs corresponding to Dicer. For Western analysis, T7-tagged proteins were immunoprecipitated from whole cell lysates in IP buffer using anti-T7- antibody-agarose conjugates. Proteins were released from the beads by boiling in Laemmli buffer and were separated by electrophoresis on 8% SDS PAGE. Following transfer to nitrocellulose, proteins were visualized using an HRP-conjugated anti-T7 20 antibody (Novagen) and chemiluminescent detection (Supersignal, Pierce).

*RNAi of Dicer*. *Drosophila* S2 cells were transfected either with a dsRNA corresponding to mouse caspase 9 or with a mixture of two dsRNAs corresponding to *Drosophila* Dicer-1 and Dicer-2 (CG4792 and CG6493). Two days after the initial transfection, cells were again transfected with a mixture containing a GFP expression 25 plasmid and either luciferase dsRNA or GFP dsRNA as previously described<sup>6</sup>. Cells were assayed for Dicer activity or fluorescence three days after the second transfection. Quantification of fluorescent cells was done on a Coulter EPICS cell sorter after fixation. Control transfections indicated that Dicer activity was not affected by the introduction of caspase 9 dsRNA.

30

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**Example 3: A simplified method for the creation of hairpin constructs for RNA interference.**

In numerous model organisms, double stranded RNAs have been shown to cause 5 effective and specific suppression of gene function (ref. 1). This response, termed RNA interference or post-transcriptional gene silencing, has evolved into a highly effective reverse genetic tool in *C. elegans*, *Drosophila*, plants and numerous other systems. In these cases, double-stranded RNAs can be introduced by injection, transfection or feeding; however, in all cases, the response is both transient and systemic. Recently, stable 10 interference with gene expression has been achieved by expression of RNAs that form snap-back or hairpin structures (refs 2-7). This has the potential not only to allow stable silencing of gene expression but also inducible silencing as has been observed in trypanosomes and adult *Drosophila* (refs 2,4,5). The utility of this approach is somewhat hampered by the difficulties that arise in the construction of bacterial plasmids containing 15 the long inverted repeats that are necessary to provoke silencing. In a recent report, it was stated that more than 1,000 putative clones were screened to identify the desired construct (ref 7).

The presence of hairpin structures often induces plasmid rearrangement, in part due to the *E. coli* sbc proteins that recognize and cleave cruciform DNA structures (ref 8). 20 We have developed a method for the construction of hairpins that does not require cloning of inverted repeats, *per se*. Instead, the fragment of the gene that is to be silenced is cloned as a direct repeat, and the inversion is accomplished by treatment with a site-specific recombinase, either *in vitro* (or potentially *in vivo*) (see Fig 29). Following recombination, the inverted repeat structure is stable in a bacterial strain that lacks an 25 intact SBC system (DL759). We have successfully used this strategy to construct numerous hairpin expression constructs that have been successfully used to provoke gene silencing in *Drosophila* cells.

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*V. Equivalents*

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

All of the above-cited references and publications are hereby incorporated by reference.

We Claim:

1. A method for attenuating expression of a target gene in a non-embryonic cell suspended in culture, comprising introducing into the cell a double stranded RNA (dsRNA) in an amount sufficient to attenuate expression of the target gene, 5 wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the target gene.
2. A method for attenuating expression of a target gene in a mammalian cell, comprising 10 (i) activating one or both of a Dicer activity or an Argonaut activity in the cell, and (ii) introducing into the cell a double stranded RNA (dsRNA) in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions 15 to a nucleotide sequence of the target gene.
3. The method of claim 2, wherein the cell is suspended in culture.
4. The method of claim 2, wherein the cell is in a whole animal, such as a non-human 20 mammal.
5. The method of claim 1 or 2, wherein is engineered with (i) a recombinant gene encoding a Dicer activity, (ii) a recombinant gene encoding an Argonaut activity, or (iii) both. 25
6. The method of claim 5, wherein the recombinant gene encodes a protein which includes an amino acid sequence at least 50 percent identical to SEQ ID No. 2 or 4 or the Argonaut sequence shown in Figure 24.
- 30 7. The method of claim 5, wherein the recombinant gene includes a coding sequence hybridizes under wash conditions of 2 x SSC at 22°C to SEQ ID No. 1 or 3.
8. The method of claim 1 or 2, wherein an endogenous Dicer gene or Argonaut gene is activated.
- 35 9. The method of claim 1 or 2, wherein the target gene is an endogenous gene of the cell.

10. The method of claim 1 or 2, wherein the target gene is an heterologous gene relative to the genome of the cell, such as a pathogen gene.
- 5 11. The method of claim 1 or 2, wherein the cell is treated with an agent that inhibits protein kinase RNA-activated (PKR) apoptosis, such as by treatment with agents which inhibit expression of PKR, cause its destruction, and/or inhibit the kinase activity of PKF.
- 10 12. The method of claim 1 or 2, wherein the cell is a primate cell, such as a human cell.
13. The method of claim 1 or 2, wherein the dsRNA is at least 50 nucleotides in length.
- 15 14. The method of claim 13, wherein the dsRNA is 400-800 nucleotides in length.
15. The method of claim 13, wherein the dsRNA is 400-800 nucleotides in length.
- 20 16. An assay for identifying nucleic acid sequences responsible for conferring a particular phenotype in a cell, comprising
  - (i) constructing a variegated library of nucleic acid sequences from a cell in an orientation relative to a promoter to produce double stranded DNA;
  - (ii) introducing the variegated dsRNA library into a culture of target cells, which cells have an activated Dicer activity or Argonaut activity;
  - 25 (iii) identifying members of the library which confer a particular phenotype on the cell, and identifying the sequence from a cell which correspond, such as being identical or homologous, to the library member.
- 30 17. A method of conducting a drug discovery business comprising:
  - (i) identifying, by the assay of claim 16, a target gene which provides a phenotypically desirable response when inhibited by RNAi;
  - (ii) identifying agents by their ability to inhibit expression of the target gene or the activity of an expression product of the target gene;
  - 35 (iii) conducting therapeutic profiling of agents identified in step (b), or further analogs thereof, for efficacy and toxicity in animals; and

- (iv) formulating a pharmaceutical preparation including one or more agents identified in step (iii) as having an acceptable therapeutic profile.
18. The method of claim 17, including an additional step of establishing a distribution system for distributing the pharmaceutical preparation for sale, and may optionally 5 include establishing a sales group for marketing the pharmaceutical preparation.
19. A method of conducting a target discovery business comprising:
  - (i) identifying, by the assay of claim 16, a target gene which provides a phenotypically desirable response when inhibited by RNAi;
  - 10 (ii) (optionally) conducting therapeutic profiling of the target gene for efficacy and toxicity in animals; and
  - (iii). licensing, to a third party, the rights for further drug development of inhibitors of the target gene.

15

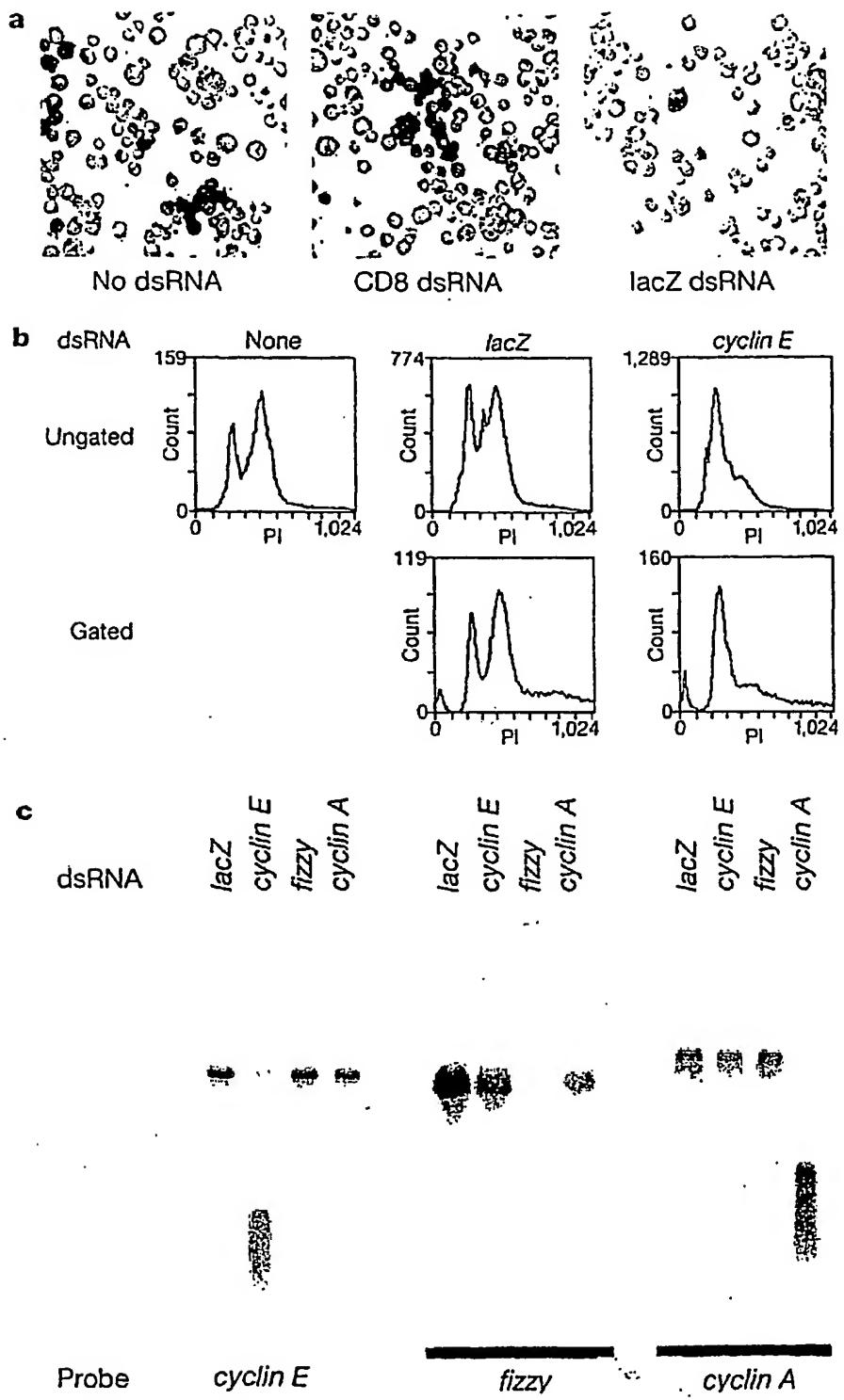


Figure 2

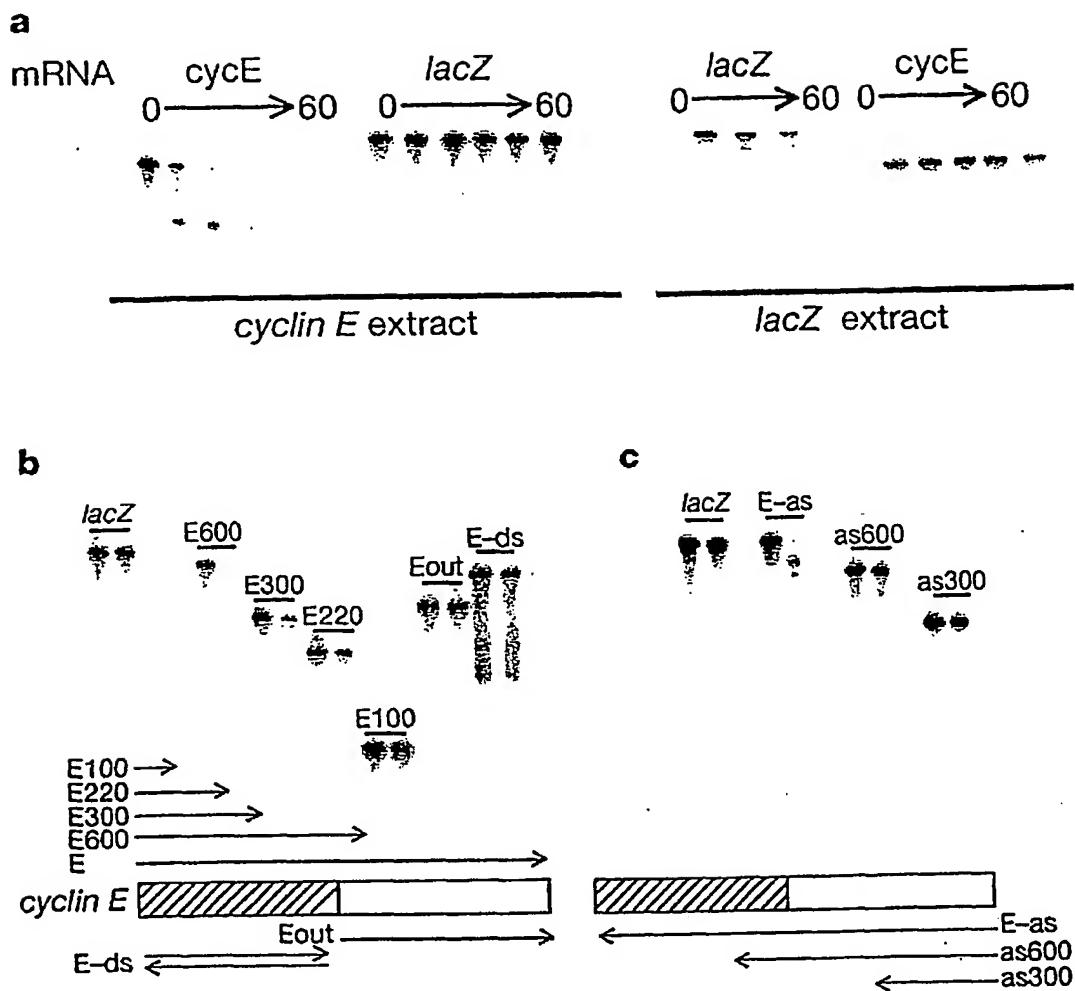


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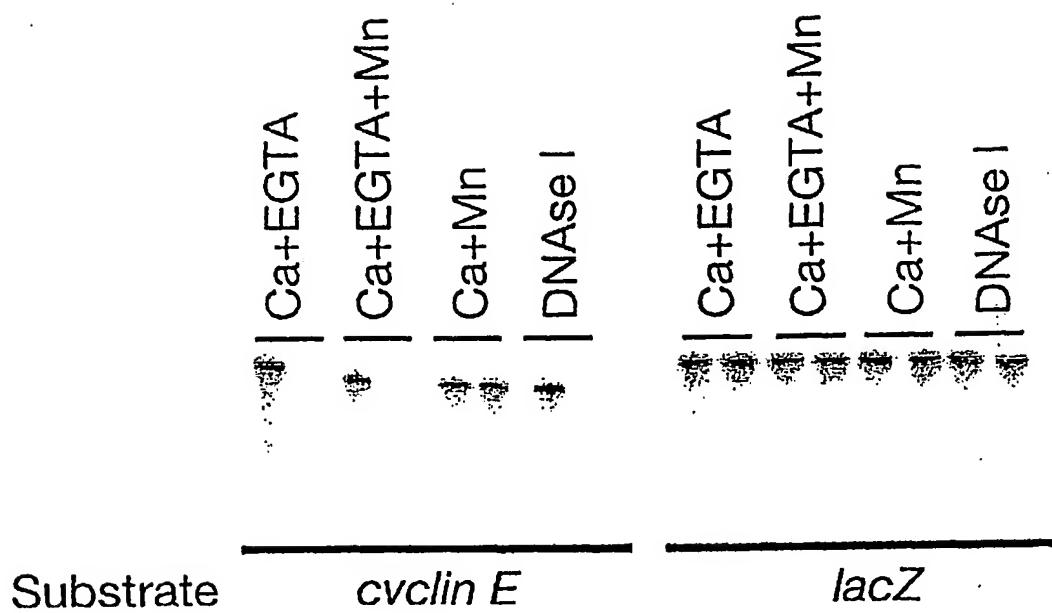


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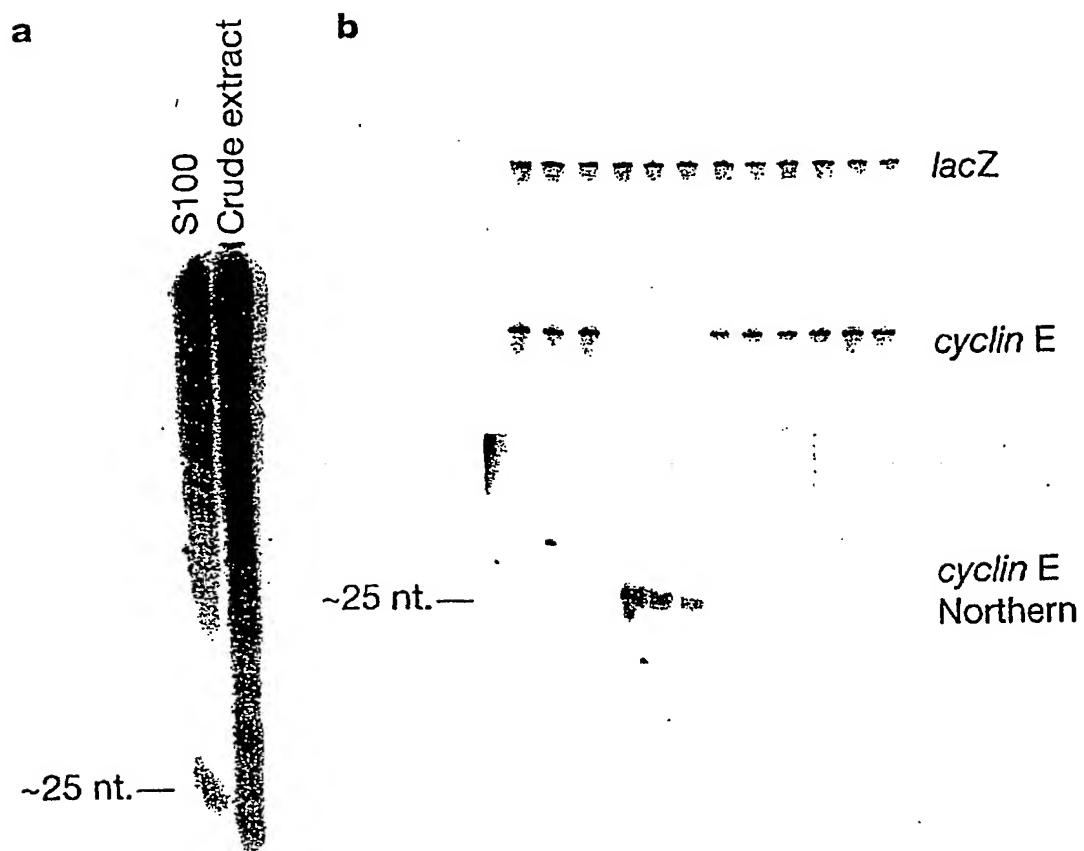


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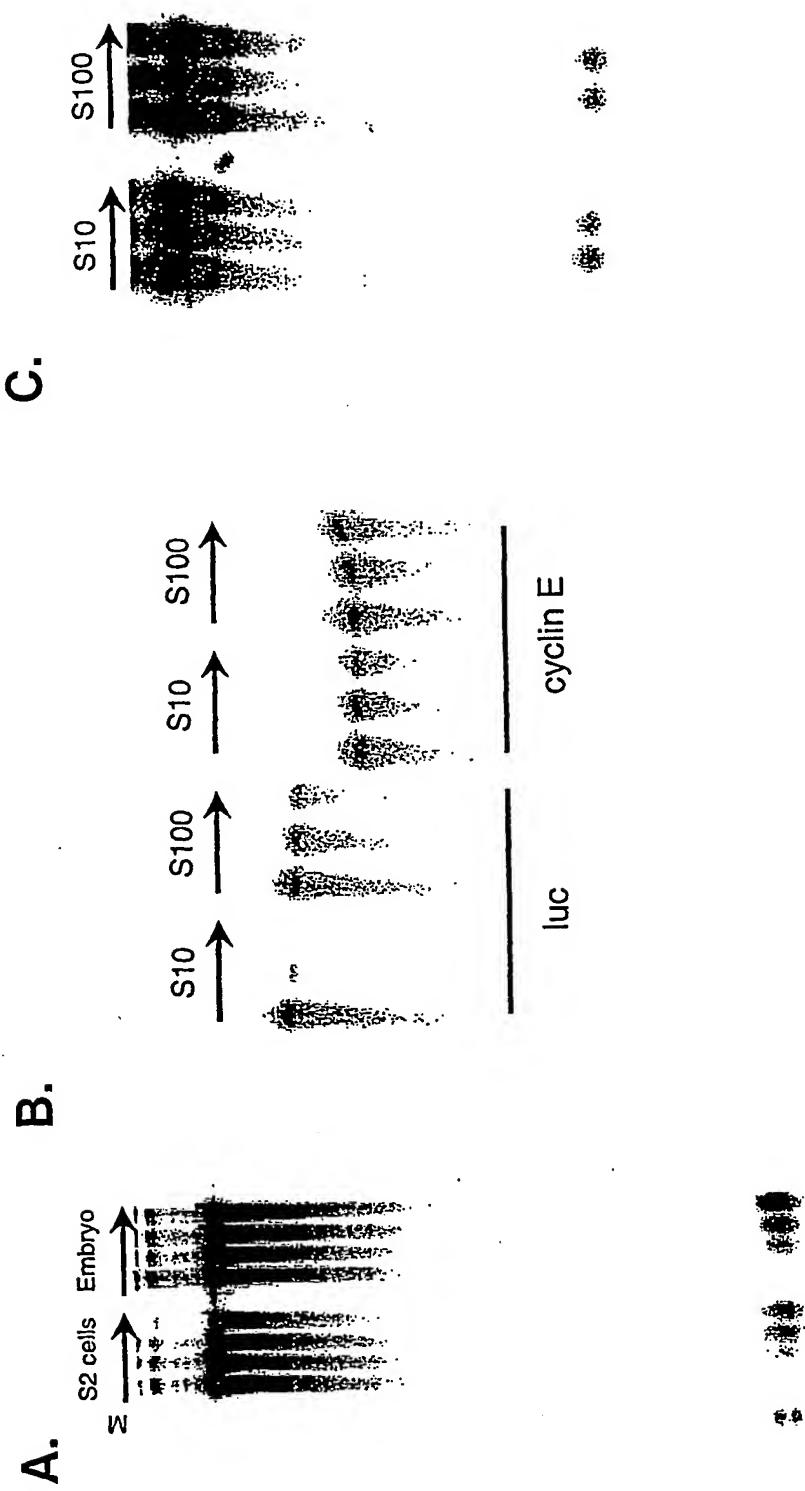


Figure 6a-c

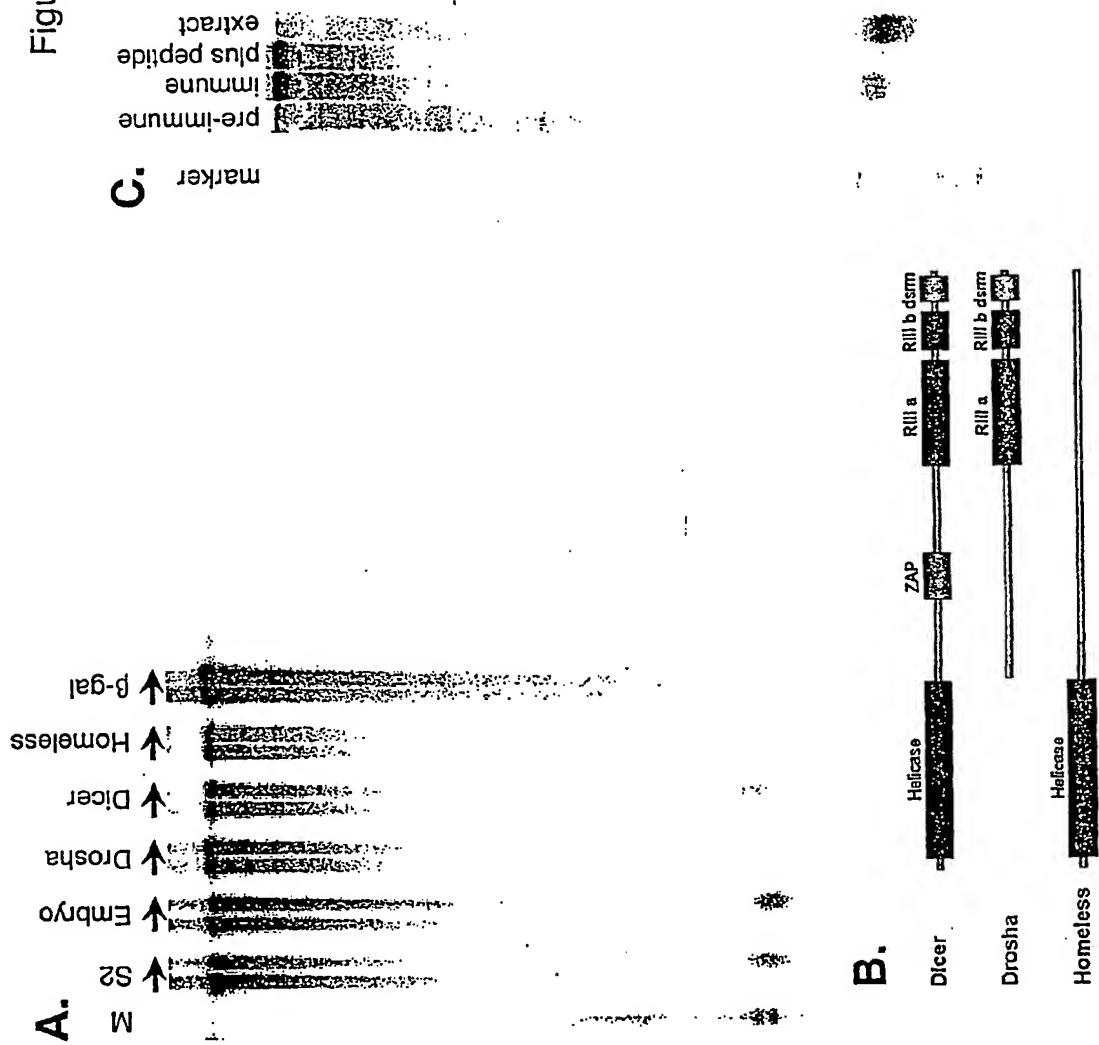
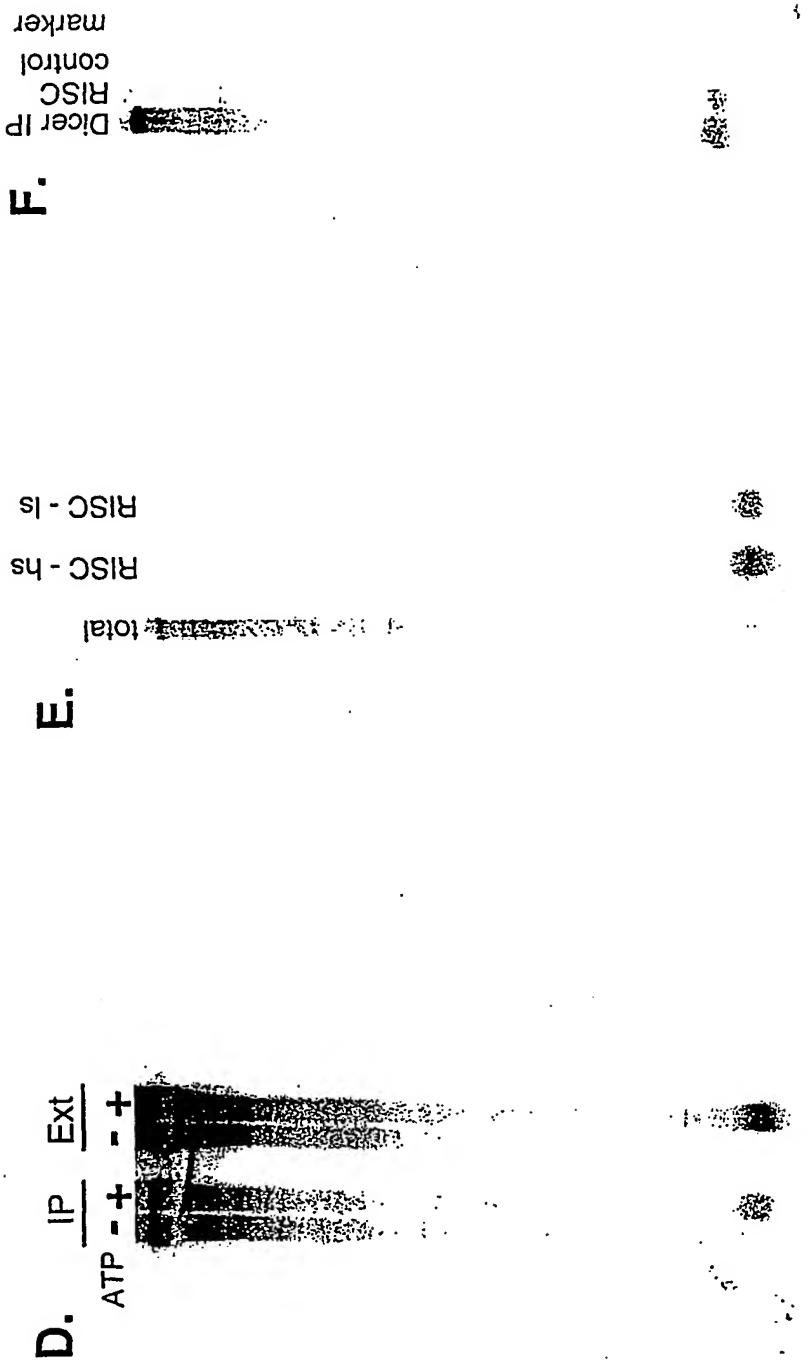


Figure 6d-f



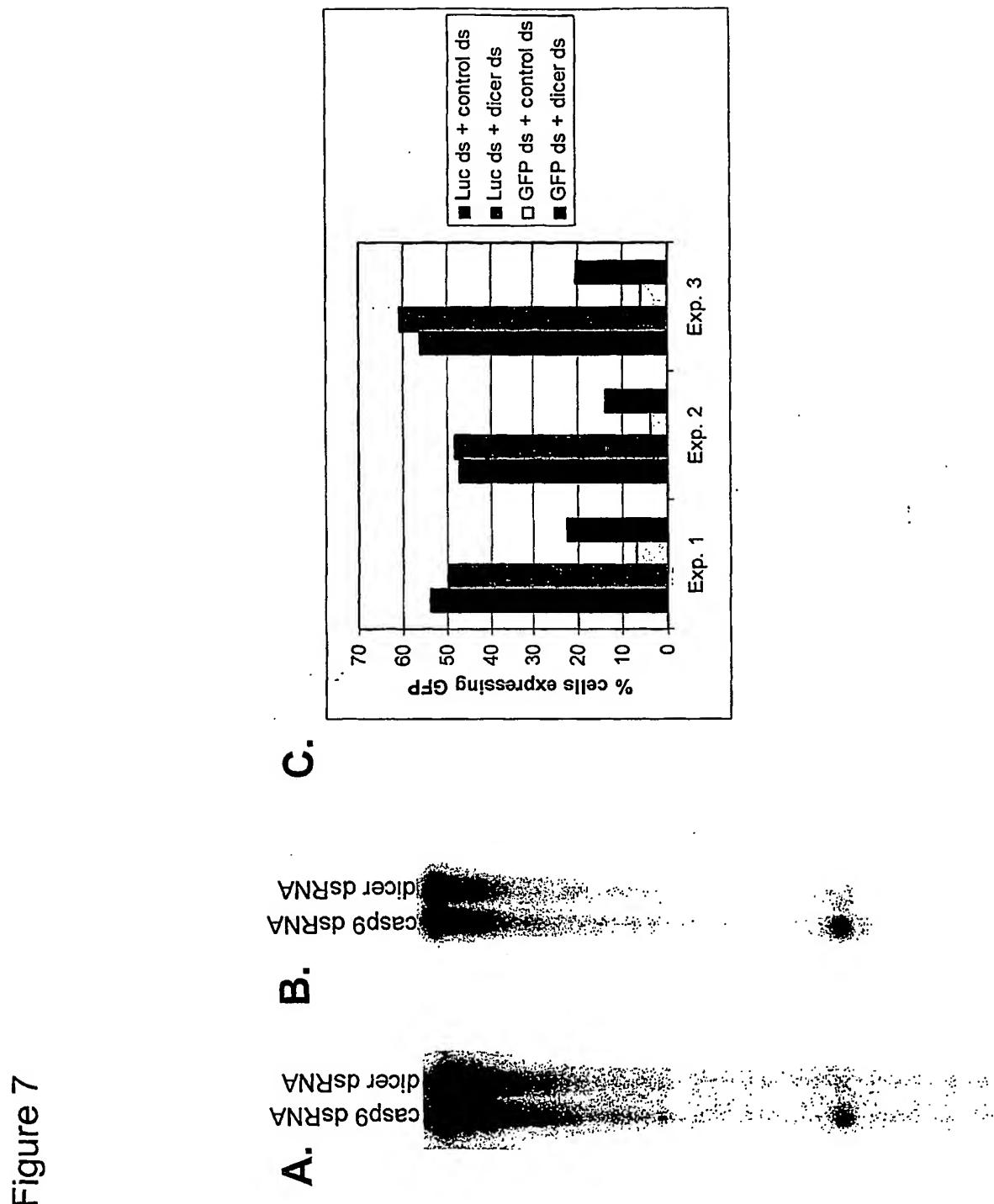
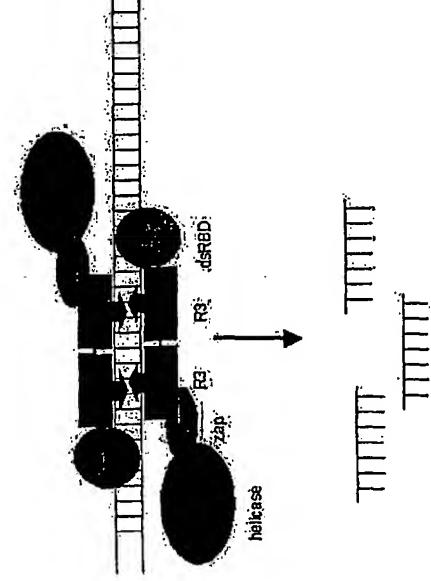
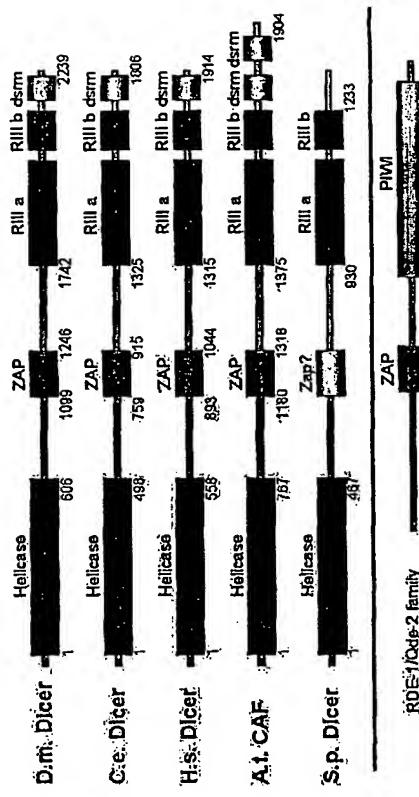


Figure 8A, B

A



B



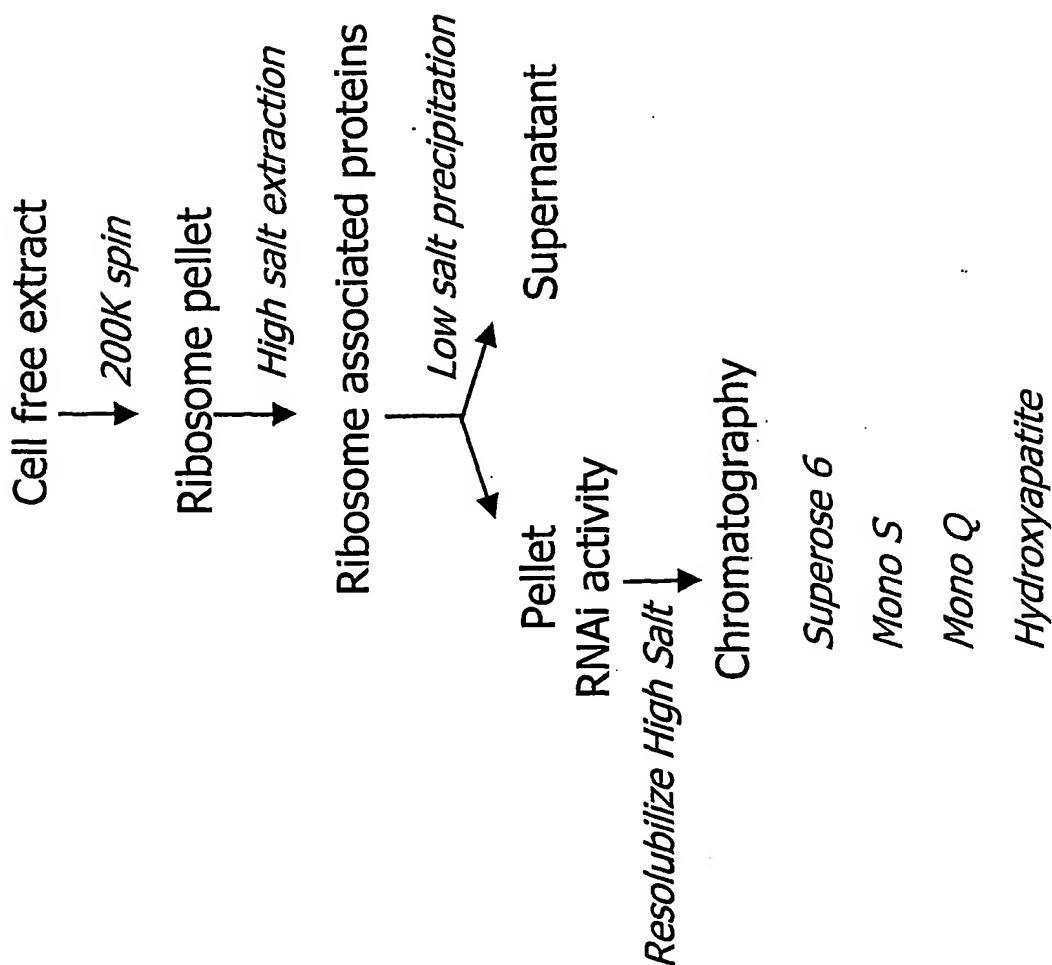
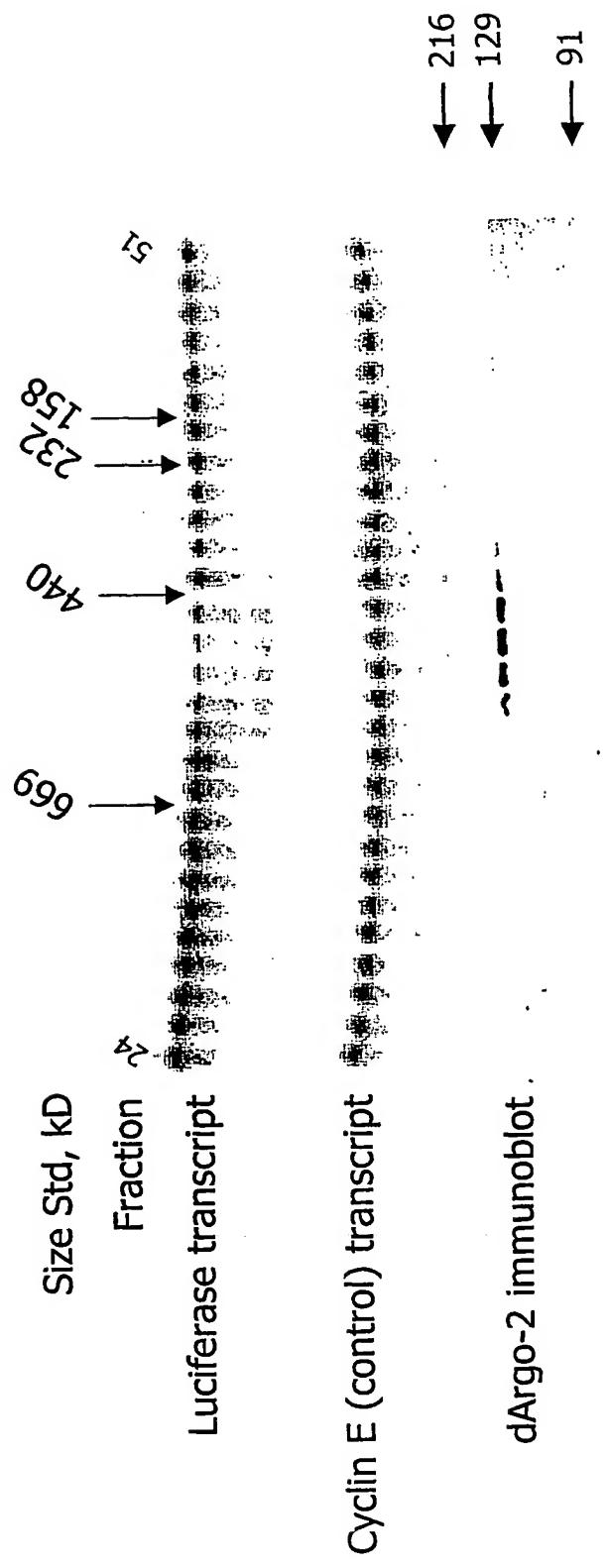


Figure 9

Figure 10



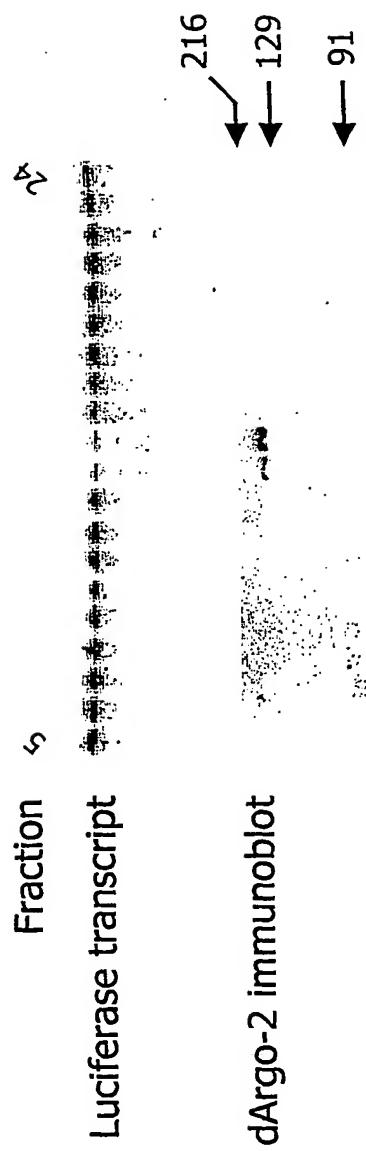


Figure 11

Figure 12

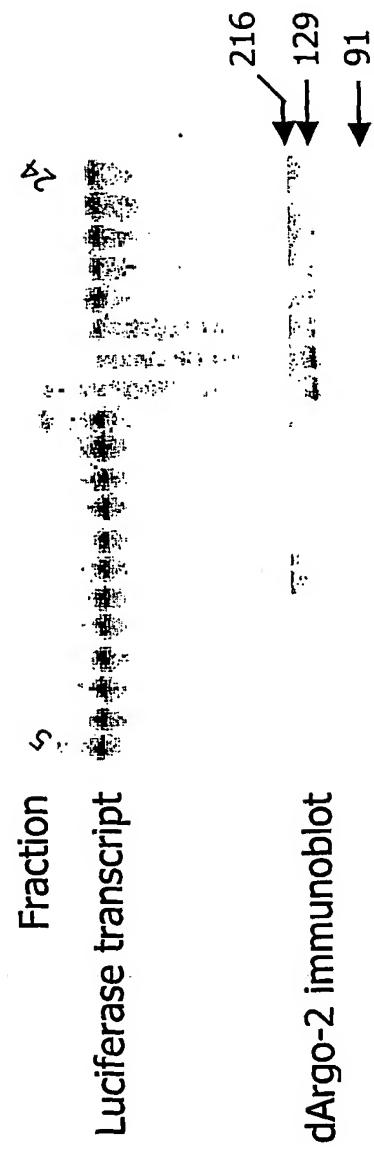
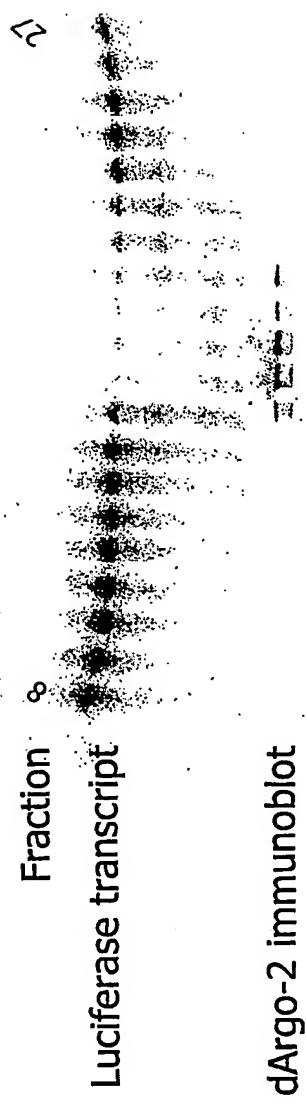


Figure 13



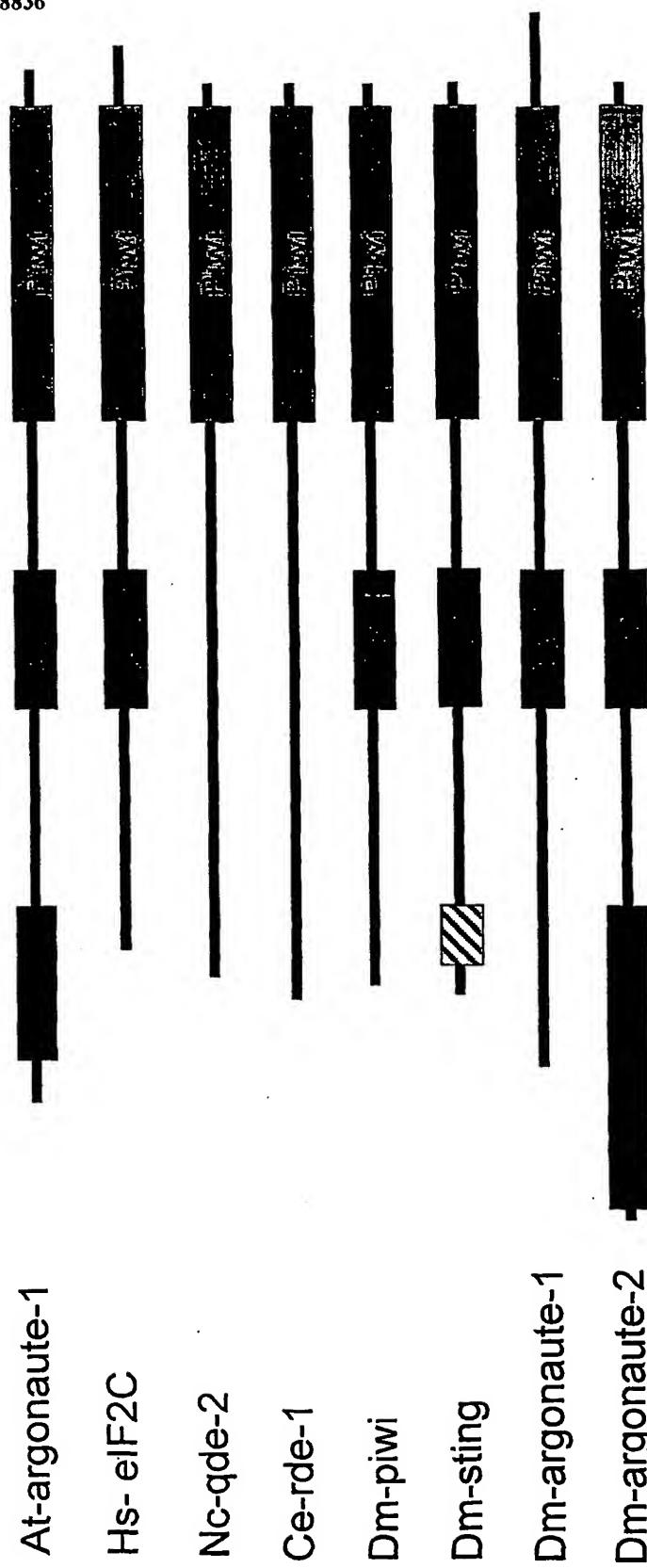
**Figure 14**



Figure 15

Figure 16

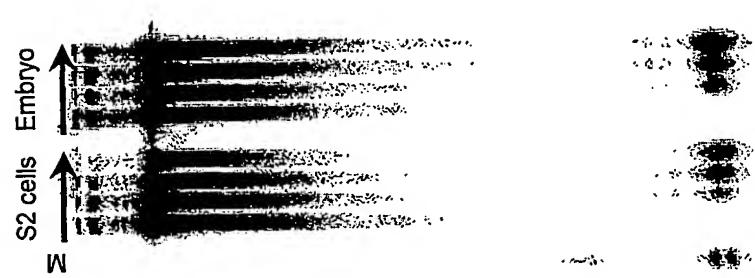
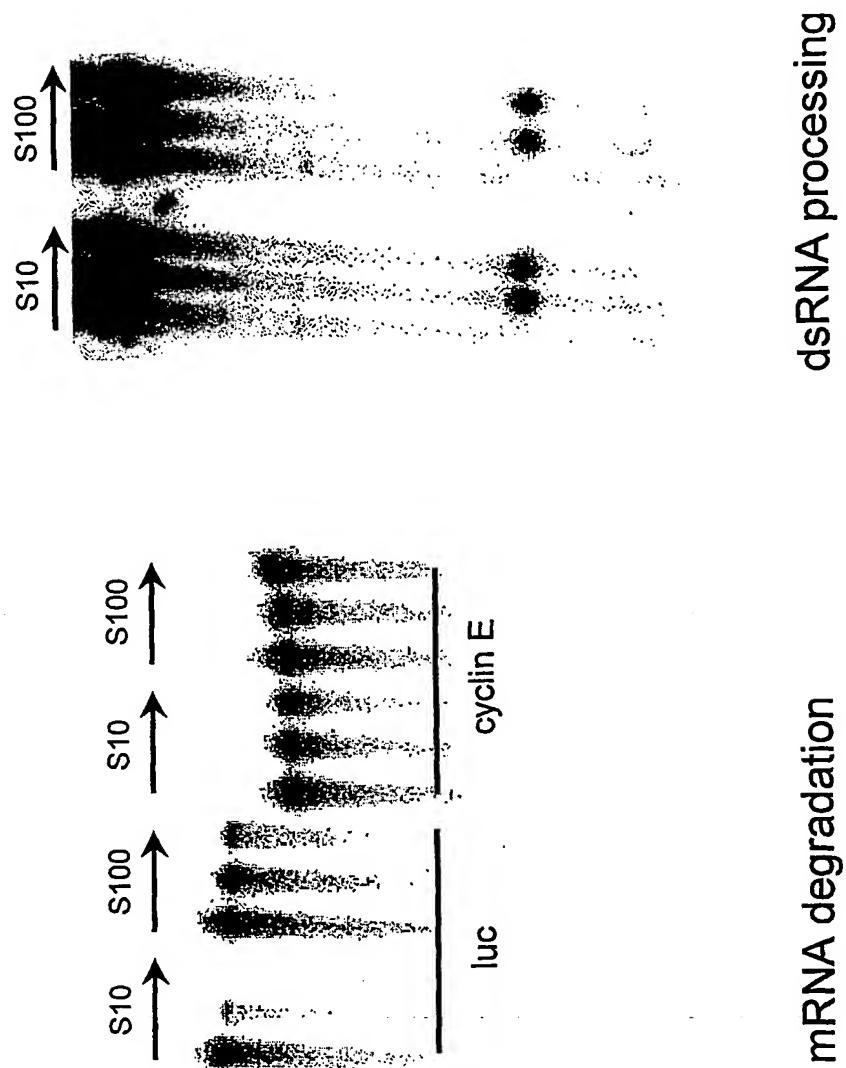
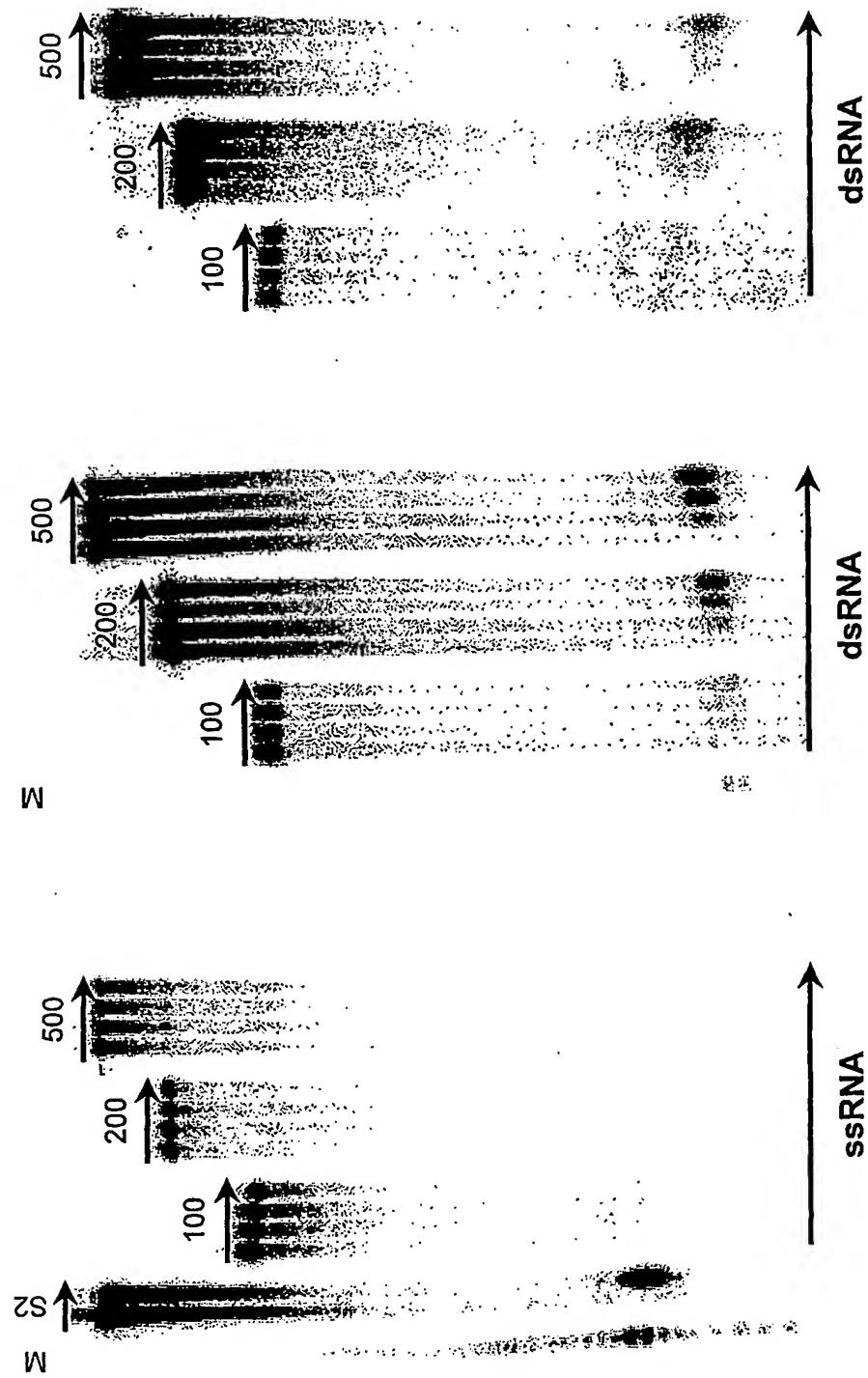


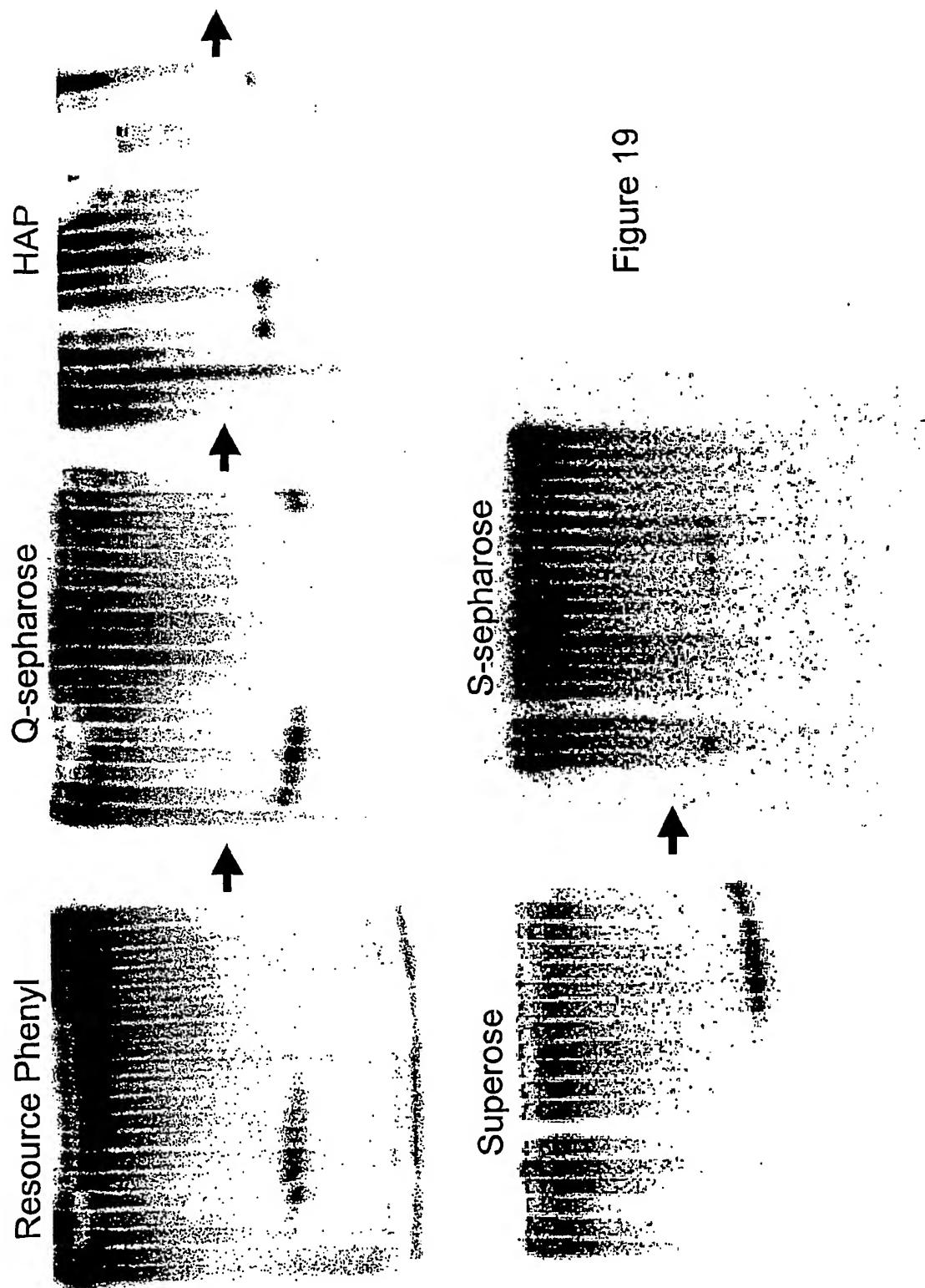
Figure 17



dsRNA processing  
mRNA degradation

Figure 18





Purification of the 22-mer generating enzyme

Figure 20

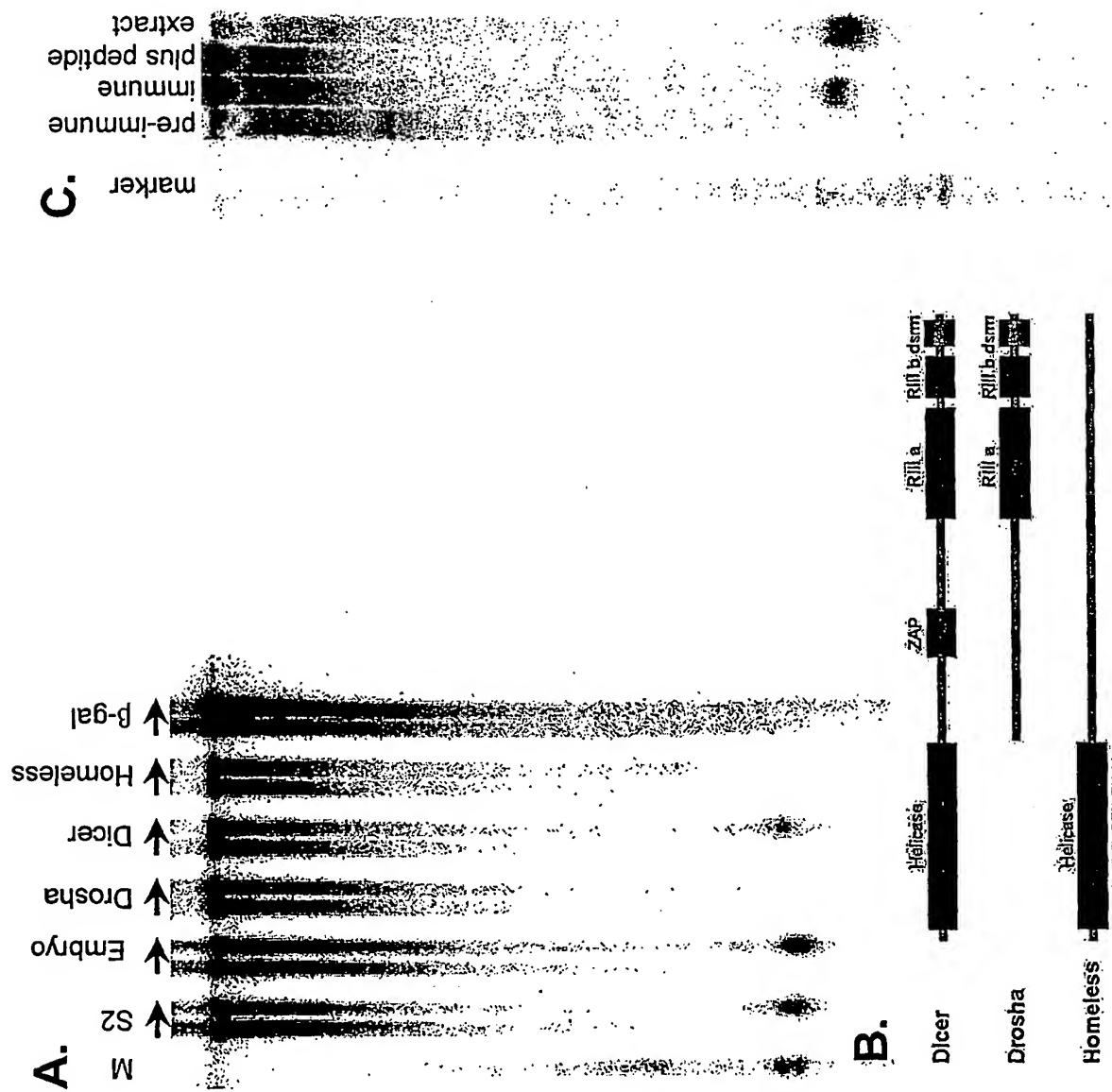


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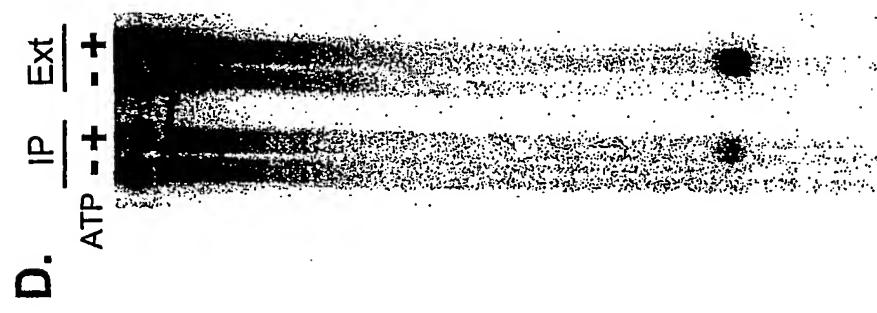
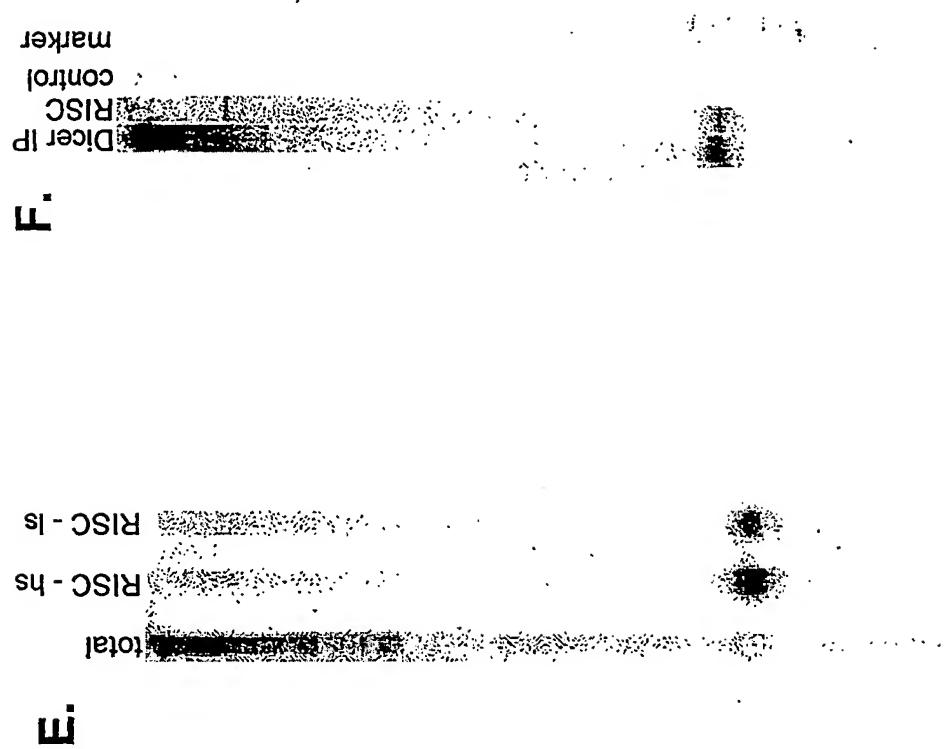


Figure 22



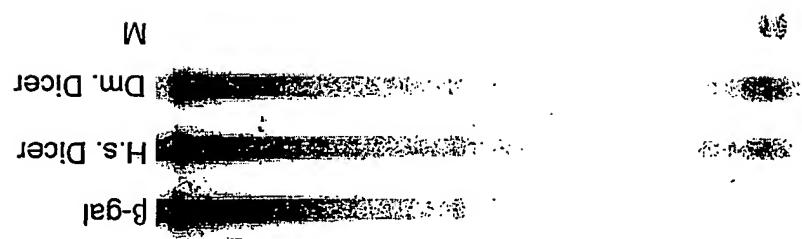
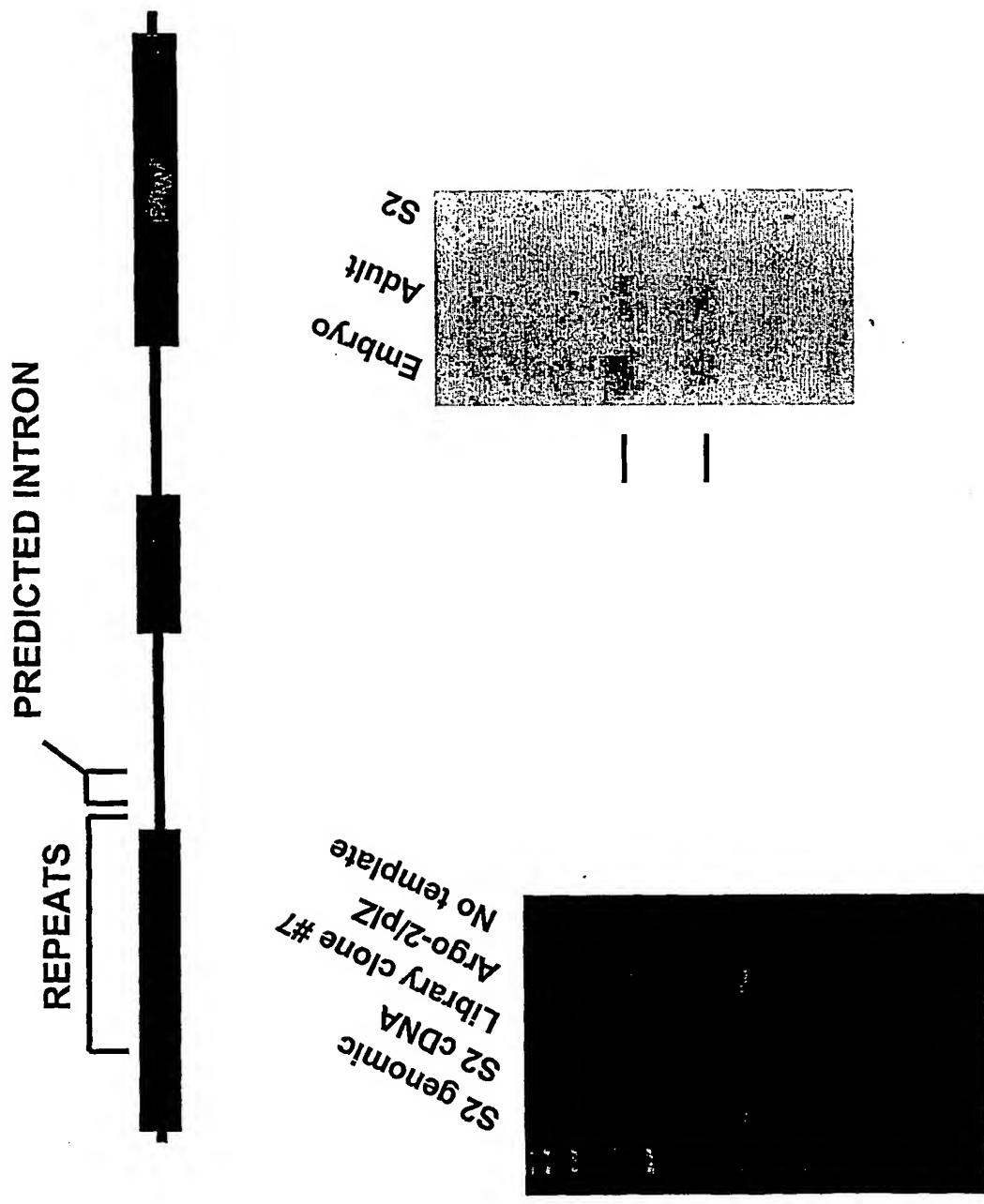


Figure 23

Figure 24

Figure 25



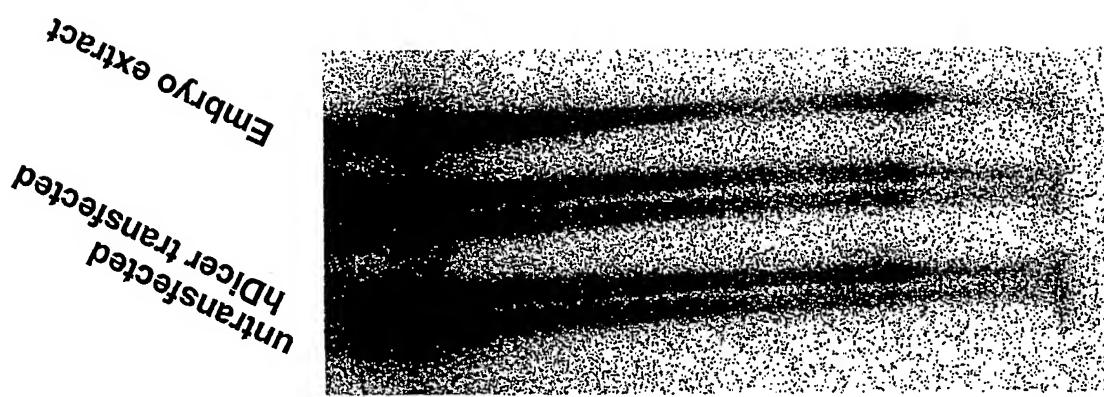


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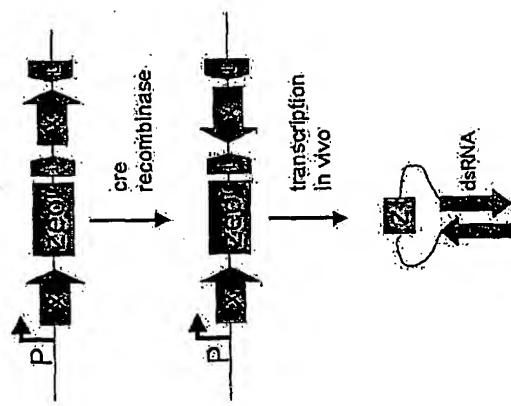


Figure 27

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gat gag tgt cat ctt gca atc cta gac cac ccc tat cga gaa ttg atg 576  
Asp Glu Cys His Leu Ala Ile Leu Asp His Pro Tyr Arg Glu Phe Met  
180 185 190

aag ctc tgt gaa att tgt cca tca tgt cct cgc att ttg gga cta act 624  
Lys Leu Cys Glu Ile Cys Pro Ser Cys Pro Arg Ile Leu Gly Leu Thr  
195 200 205

gct tcc att tta aat ggg aaa tgg gat cca gag gat ttg gaa gaa aag 672  
Ala Ser Ile Leu Asn Gly Lys Trp Asp Pro Glu Asp Leu Glu Glu Lys  
210 215 220

ttt cag aaa cta gag aaa att ctt aag agt aat gct gaa act gca act 720  
Phe Gln Lys Leu Glu Lys Ile Leu Lys Ser Asn Ala Glu Thr Ala Thr  
225 230 235 240

gac ctg gtg gtc tta gac agg tat act tct cag cca tgt gag att gtg 768  
Asp Leu Val Val Leu Asp Arg Tyr Thr Ser Gln Pro Cys Glu Ile Val  
245 250 255

gtg gat tgt gga cca ttt act gac aga agt ggg ctt tat gaa aga ctg 816  
Val Asp Cys Gly Pro Phe Thr Asp Arg Ser Gly Leu Tyr Glu Arg Leu  
260 265 270

ctg atg gaa tta gaa gaa gca ctt aat ttt atc aat gat tgt aat ata 864  
Leu Met Glu Leu Glu Ala Leu Asn Phe Ile Asn Asp Cys Asn Ile  
275 280 285

tct gta cat tca aaa gaa aga gat tct act tta att tcg aaa cag ata 912  
Ser Val His Ser Lys Glu Arg Asp Ser Thr Leu Ile Ser Lys Gln Ile  
290 295 300

cta tca gac tgt cgt gcc gta ttg gta gtt ctg gga ccc tgg tgt gca 960  
Leu Ser Asp Cys Arg Ala Val Leu Val Val Leu Gly Pro Trp Cys Ala  
305 310 315 320

gat aaa gta gct gga atg atg gta aga gaa cta cag aaa tac atc aaa 1008

Asp Lys Val Ala Gly Met Met Val Arg Glu Leu Gin Lys Tyr Ile Lys

325 330 335

cat gag caa gag gag ctg cac agg aaa ttt tta ttg ttt aca gac act 1056

His Glu Gln Glu Glu Leu His Arg Lys Phe Leu Leu Phe Thr Asp Thr

340 345 350

ttc cta agg aaa ata cat gca cta tgt gaa gag cac ttc tca cct gcc 1104

Phe Leu Arg Lys Ile His Ala Leu Cys Glu Glu His Phe Ser Pro Ala

355 360 365

tca ctt gac ctg aaa ttt gta act cct aaa gta atc aaa ctg ctc gaa 1152

Ser Leu Asp Leu Lys Phe Val Thr Pro Lys Val Ile Lys Leu Leu Glu

370 375 380

atc tta cgc aaa tat aaa cca tat gag cga cac agt ttt gaa agc gtt 1200

Ile Leu Arg Lys Tyr Lys Pro Tyr Glu Arg His Ser Phe Glu Ser Val

385 390 395 400

gag tgg tat aat aat aga aat cag gat aat tat gtg tca tgg agt gat 1248

Glu Trp Tyr Asn Asn Arg Asn Gln Asp Asn Tyr Val Ser Trp Ser Asp

405 410 415

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Ser Glu Asp Asp Asp Glu Asp Glu Glu Ile Glu Glu Lys Glu Lys Pro

420 425 430

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Glu Thr Asn Phe Pro Ser Pro Phe Thr Asn Ile Leu Cys Gly Ile Ile

435 440 445

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450 455 460

gaa gct ggc aaa caa gat cca gag ctg gct tat atc agt agc aat ttc 1440

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465 470 475 480

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Ile Thr Gly His Gly Ile Gly Lys Asn Gln Pro Arg Asn Asn Thr Met

485 490 495

gaa gca gaa ttc aga aaa cag gaa gag gta ctt agg aaa ttt cga gca 1536

Glu Ala Glu Phe Arg Lys Gln Glu Glu Val Leu Arg Lys Phe Arg Ala

500 505 510

cat gag acc aac ctg ctt att gca aca agt att gta gaa gag ggt gtt 1584

His Glu Thr Asn Leu Leu Ile Ala Thr Ser Ile Val Glu Glu Gly Val

515 520 525

gat ata cca aaa tgc aac ttg gtg gtt cgt ttt gat ttg ccc aca gaa 1632

Asp Ile Pro Lys Cys Asn Leu Val Val Arg Phe Asp Leu Pro Thr Glu

530 535 540

tat cga tcc tat gtt caa tct aaa gga aga gca agg gca ccc atc tct 1680

Tyr Arg Ser Tyr Val Gln Ser Lys Gly Arg Ala Arg Ala Pro Ile Ser

545 550 555 560

aat tat ata atg tta gcg gat aca gac aaa ata aaa agt ttt gaa gaa 1728

Asn Tyr Ile Met Leu Ala Asp Thr Asp Lys Ile Lys Ser Phe Glu Glu

565 570 575

gac ctt aaa acc tac aaa got att gaa aag atc ttg aga aac aag tgt 1776

Asp Leu Lys Thr Tyr Lys Ala Ile Glu Lys Ile Leu Arg Asn Lys Cys

580 585 590

tcc aag tcg gtt gat act ggt gag act gac att gat cct gtc atg gat 1824

Ser Lys Ser Val Asp Thr Gly Glu Thr Asp Ile Asp Pro Val Met Asp

595 600 605

gat gat cac gtt ttc cca cca tat gtg ttg agg cct gac gat ggt ggt 1872

Asp Asp His Val Phe Pro Pro Tyr Val Leu Arg Pro Asp Asp Gly Gly

610 615 620

cca cga gtc aca atc aac acg gcc att gga cac atc aat aga tac tgt 1920  
Pro Arg Val Thr Ile Asn Thr Ala Ile Gly His Ile Asn Arg Tyr Cys

625 630 635 640

gtc aga tta cca agt gat ccg ttt act cat cta gct cct aaa tgc aga 1968  
Ala Arg Leu Pro Ser Asp Pro Phe Thr His Leu Ala Pro Lys Cys Arg

645 650 655

acc cga gag ttg cct gat ggt aca ttt tat tca act ctt tat ctg cca 2016  
Thr Arg Glu Leu Pro Asp Gly Thr Phe Tyr Ser Thr Leu Tyr Leu Pro

660 665 670

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Ile Asn Ser Pro Leu Arg Ala Ser Ile Val Gly Pro Pro Met Ser Cys

675 680 685

gta cga ttg gct gaa aga gtt gtc gct ctc att tgc tgt gag aaa ctg 2112  
Val Arg Leu Ala Glu Arg Val Val Ala Leu Ile Cys Cys Glu Lys Leu

690 695 700

cac aaa att ggc gaa ctg gat gac cat ttg atg cca gtt ggg aaa gag 2160  
His Lys Ile Gly Glu Leu Asp Asp His Leu Met Pro Val Gly Lys Glu

705 710 715 720

act gtt aaa tat gaa gag gag ctt gat ttg cat gat gaa gaa gag acc 2208  
Thr Val Lys Tyr Glu Glu Glu Leu Asp Leu His Asp Glu Glu Glu Thr

725 730 735

agt gtt cca gga aga cca ggt tcc acg aaa cga agg cag tgc tac cca 2256  
Ser Val Pro Gly Arg Pro Gly Ser Thr Lys Arg Arg Gln Cys Tyr Pro

740 745 750

aaa gca att cca gag tgt ttg agg gat agt tat ccc aga cct gat cag 2304  
Lys Ala Ile Pro Glu Cys Leu Arg Asp Ser Tyr Pro Arg Pro Asp Gln

755 760 765

ccc tgt tac ctg tat gtg ata gga atg gtt tta act aca cct tta cct 2352

Pro Cys Tyr Leu Tyr Val Ile Gly Met Val Leu Thr Thr Pro Leu Pro

770 775 780

gat gaa ctc aac ttt aga agg cgg aag ctc tat cct cct gaa gat acc 2400

Asp Glu Leu Asn Phe Arg Arg Arg Lys Leu Tyr Pro Pro Glu Asp Thr

785 790 795 800

aca aga tgc ttt gga ata ctg acg gcc aaa ccc ata cct cag att cca 2448

Thr Arg Cys Phe Gly Ile Leu Thr Ala Lys Pro Ile Pro Gln Ile Pro

805 810 815

cac ttt cct gtg tac aca cgc tct gga gag gtt acc ata tcc att gag 2496

His Phe Pro Val Tyr Thr Arg Ser Gly Glu Val Thr Ile Ser Ile Glu

820 825 830

ttg aag aag tct ggt ttc atg ttg tot cta caa atg ctt gag ttg att 2544

Leu Lys Lys Ser Gly Phe Met Leu Ser Leu Gln Met Leu Glu Leu Ile

835 840 845

aca aga ctt cac cag tat ata ttc tca cat att ctt cgg ctt gaa aaa 2592

Thr Arg Leu His Gln Tyr Ile Phe Ser His Ile Leu Arg Leu Glu Lys

850 855 860

cct gca cta gaa ttt aaa cct aca gac gct gat tca gca tac tgt gtt 2640

Pro Ala Leu Glu Phe Lys Pro Thr Asp Ala Asp Ser Ala Tyr Cys Val

865 870 875 880

cta cct ctt aat gtt gtt aat gac tcc agc act ttg gat att gac ttt 2688

Leu Pro Leu Asn Val Val Asn Asp Ser Ser Thr Leu Asp Ile Asp Phe

885 890 895

aaa ttc atg gaa gat att gag aag tct gaa gct cgc ata ggc att ccc 2736

Lys Phe Met Glu Asp Ile Glu Lys Ser Glu Ala Arg Ile Gly Ile Pro

900 905 910

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 Ser Thr Lys Tyr Thr Lys Glu Thr Pro Phe Val Phe Lys Leu Glu Asp  
 915 920 925

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 Tyr Gln Asp Ala Val Ile Ile Pro Arg Tyr Arg Asn Phe Asp Gln Pro  
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cat cga ttt tat gta gct gat gtg tac act gat ctt acc cca ctc agt 2880  
 His Arg Phe Tyr Val Ala Asp Val Tyr Thr Asp Leu Thr Pro Leu Ser  
 945 950 955 960

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 Lys Phe Pro Ser Pro Glu Tyr Glu Thr Phe Ala Glu Tyr Tyr Lys Thr  
 965 970 975

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 Lys Tyr Asn Leu Asp Leu Thr Asn Leu Asn Gln Pro Leu Leu Asp Val  
 980 985 990

gac cac aca tct tca aga ctt aat ctt ttg aca cct cga cat ttg aat 3024  
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Val Cys Leu Pro Ser Ile Leu Tyr Arg Leu His Cys Leu Leu Thr

1055 1060 1065

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1070 1075 1080

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Val Arg Ser Leu Pro Ala Asp Phe Arg Tyr Pro Asn Leu Asp Phe

1085 1090 1095

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Gly Trp Lys Lys Ser Ile Asp Ser Lys Ser Phe Ile Ser Ile Ser

1100 1105 1110

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Asn Ser Ser Ser Ala Glu Asn Asp Asn Tyr Cys Lys His Ser Thr

1115 1120 1125

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Ile Val Pro Glu Asn Ala Ala His Gln Gly Ala Asn Arg Thr Ser

1130 1135 1140

tct cta gaa aat cat gac caa atg tct gtg aac tgc aga acg ttg 3474

Ser Leu Glu Asn His Asp Gln Met Ser Val Asn Cys Arg Thr Leu

1145 1150 1155

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Leu Ser Glu Ser Pro Gly Lys Leu His Val Glu Val Ser Ala Asp

1160 1165 1170

ctt aca gca att aat ggt ctt tct tac aat caa aat ctc gcc aat 3564

Leu Thr Ala Ile Asn Gly Leu Ser Tyr Asn Gln Asn Leu Ala Asn

1175 1180 1185

ggc agt tat gat tta gct aac aga gac ttt tgc caa gga aat cag 3609

Gly Ser Tyr Asp Leu Ala Asn Arg Asp Phe Cys Gln Gly Asn Gln

1190 1195 1200

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1205 1210 1215

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1295 1300 1305

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Asp Ala His Glu Gly Arg Leu Ser Tyr Met Arg Ser Lys Lys Val

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Pro Pro Gly Tyr Val Val Asn Gln Asp Lys Ser Asn Thr Asp Lys

1385 1390 1395

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Lys Leu Asp Glu Asp Tyr Glu Glu Glu Asp Glu Glu Glu Ser

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1430 1435 1440

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1445 1450 1455

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Leu Met Gly Ser Gly Ala Phe Val Lys Lys Ile Ser Leu Ser Pro

1460 1465 1470

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 Cys Val Glu Ala Leu Leu Gly Cys Tyr Leu Thr Ser Cys Gly Glu  
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1670 1675 1680

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1715 1720 1725

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1730 1735 1740

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1760 1765 1770

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1805 1810 1815

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1835 1840 1845

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1865 1870 1875

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1880 1885 1890

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1895 1900 1905

aga gcc ctc cga agc ctc aaa gct aat caa cct cag gtt ccc aat 5769  
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Ser

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35 40 45

Val Glu Leu Leu Glu Ala Ala Leu Asp His Asn Thr Ile Val Cys Leu  
50 55 60

Asn Thr Gly Ser Gly Lys Thr Phe Ile Ala Ser Thr Thr Leu Leu Lys

65 70 75 80

Ser Cys Leu Tyr Leu Asp Leu Gly Glu Thr Ser Ala Arg Asn Gly Lys

85 90 95

Arg Thr Val Phe Leu Val Asn Ser Ala Asn Gln Val Ala Gln Gln Val

100 105 110

Ser Ala Val Arg Thr His Ser Asp Leu Lys Val Gly Glu Tyr Ser Asn

115 120 125

Leu Glu Val Asn Ala Ser Trp Thr Lys Glu Arg Trp Asn Gln Glu Phe

130 135 140

Thr Lys His Gln Val Leu Ile Met Thr Cys Tyr Val Ala Leu Asn Val

145 150 155 160

Leu Lys Asn Gly Tyr Leu Ser Leu Ser Asp Ile Asn Leu Leu Val Phe

165 170 175

Asp Glu Cys His Leu Ala Ile Leu Asp His Pro Tyr Arg Glu Phe Met

180 185 190

Lys Leu Cys Glu Ile Cys Pro Ser Cys Pro Arg Ile Leu Gly Leu Thr

195 200 205

Ala Ser Ile Leu Asn Gly Lys Trp Asp Pro Glu Asp Leu Glu Glu Lys

210 215 220

Phe Gln Lys Leu Glu Lys Ile Leu Lys Ser Asn Ala Glu Thr Ala Thr

225 230 235 240

Asp Leu Val Val Leu Asp Arg Tyr Thr Ser Gln Pro Cys Glu Ile Val

245 250 255

Val Asp Cys Gly Pro Phe Thr Asp Arg Ser Gly Leu Tyr Glu Arg Leu

260 265 270

Leu Met Glu Leu Glu Ala Leu Asn Phe Ile Asn Asp Cys Asn Ile

275 280 285

Ser Val His Ser Lys Glu Arg Asp Ser Thr Leu Ile Ser Lys Gln Ile

290 295 300

Leu Ser Asp Cys Arg Ala Val Leu Val Val Leu Gly Pro Trp Cys Ala

305 310 315 320

Asp Lys Val Ala Gly Met Met Val Arg Glu Leu Gln Lys Tyr Ile Lys

325 330 335

His Glu Gln Glu Glu Leu His Arg Lys Phe Leu Leu Phe Thr Asp Thr

340 345 350

Phe Leu Arg Lys Ile His Ala Leu Cys Glu Glu His Phe Ser Pro Ala

355 360 365

Ser Leu Asp Leu Lys Phe Val Thr Pro Lys Val Ile Lys Leu Leu Glu  
370            375            380

Ile Leu Arg Lys Tyr Lys Pro Tyr Glu Arg His Ser Phe Glu Ser Val  
385            390            395            400

Glu Trp Tyr Asn Asn Arg Asn Gln Asp Asn Tyr Val Ser Trp Ser Asp  
405            410            415

Ser Glu Asp Asp Asp Glu Asp Glu Glu Ile Glu Glu Lys Glu Lys Pro  
420            425            430

Glu Thr Asn Phe Pro Ser Pro Phe Thr Asn Ile Leu Cys Gly Ile Ile  
435            440            445

Phe Val Glu Arg Arg Tyr Thr Ala Val Val Leu Asn Arg Leu Ile Lys  
450            455            460

Glu Ala Gly Lys Gln Asp Pro Glu Leu Ala Tyr Ile Ser Ser Asn Phe  
465            470            475            480

Ile Thr Gly His Gly Ile Gly Lys Asn Gln Pro Arg Asn Asn Thr Met  
485            490            495

Glu Ala Glu Phe Arg Lys Gln Glu Glu Val Leu Arg Lys Phe Arg Ala  
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His Glu Thr Asn Leu Leu Ile Ala Thr Ser Ile Val Glu Glu Gly Val

515 520 525

Asp Ile Pro Lys Cys Asn Leu Val Val Arg Phe Asp Leu Pro Thr Glu

530 535 540

Tyr Arg Ser Tyr Val Gln Ser Lys Gly Arg Ala Arg Ala Pro Ile Ser

545 550 555 560

Asn Tyr Ile Met Leu Ala Asp Thr Asp Lys Ile Lys Ser Phe Glu Glu

565 570 575

Asp Leu Lys Thr Tyr Lys Ala Ile Glu Lys Ile Leu Arg Asn Lys Cys

580 585 590

Ser Lys Ser Val Asp Thr Gly Glu Thr Asp Ile Asp Pro Val Met Asp

595 600 605

Asp Asp His Val Phe Pro Pro Tyr Val Leu Arg Pro Asp Asp Gly Gly

610 615 620

Pro Arg Val Thr Ile Asn Thr Ala Ile Gly His Ile Asn Arg Tyr Cys

625 630 635 640

Ala Arg Leu Pro Ser Asp Pro Phe Thr His Leu Ala Pro Lys Cys Arg

645 650 655

Thr Arg Glu Leu Pro Asp Gly Thr Phe Tyr Ser Thr Leu Tyr Leu Pro  
660 665 670

Ile Asn Ser Pro Leu Arg Ala Ser Ile Val Gly Pro Pro Met Ser Cys  
675 680 685

Val Arg Leu Ala Glu Arg Val Val Ala Leu Ile Cys Cys Glu Lys Leu  
690 695 700

His Lys Ile Gly Glu Leu Asp Asp His Leu Met Pro Val Gly Lys Glu  
705 710 715 720

Thr Val Lys Tyr Glu Glu Glu Leu Asp Leu His Asp Glu Glu Glu Thr  
725 730 735

Ser Val Pro Gly Arg Pro Gly Ser Thr Lys Arg Arg Gln Cys Tyr Pro  
740 745 750

Lys Ala Ile Pro Glu Cys Leu Arg Asp Ser Tyr Pro Arg Pro Asp Gln  
755 760 765

Pro Cys Tyr Leu Tyr Val Ile Gly Met Val Leu Thr Thr Pro Leu Pro  
770 775 780

Asp Glu Leu Asn Phe Arg Arg Lys Leu Tyr Pro Pro Glu Asp Thr  
785 790 795 800

Thr Arg Cys Phe Gly Ile Leu Thr Ala Lys Pro Ile Pro Gln Ile Pro

805 810 815

His Phe Pro Val Tyr Thr Arg Ser Gly Glu Val Thr Ile Ser Ile Glu

820 825 830

Leu Lys Lys Ser Gly Phe Met Leu Ser Leu Gln Met Leu Glu Leu Ile

835 840 845

Thr Arg Leu His Gln Tyr Ile Phe Ser His Ile Leu Arg Leu Glu Lys

850 855 860

Pro Ala Leu Glu Phe Lys Pro Thr Asp Ala Asp Ser Ala Tyr Cys Val

865 870 875 880

Leu Pro Leu Asn Val Val Asn Asp Ser Ser Thr Leu Asp Ile Asp Phe

885 890 895

Lys Phe Met Glu Asp Ile Glu Lys Ser Glu Ala Arg Ile Gly Ile Pro

900 905 910

Ser Thr Lys Tyr Thr Lys Glu Thr Pro Phe Val Phe Lys Leu Glu Asp

915 920 925

Tyr Gln Asp Ala Val Ile Ile Pro Arg Tyr Arg Asn Phe Asp Gln Pro

930 935 940

His Arg Phe Tyr Val Ala Asp Val Tyr Thr Asp Leu Thr Pro Leu Ser

945 950 955 960

Lys Phe Pro Ser Pro Glu Tyr Glu Thr Phe Ala Glu Tyr Tyr Lys Thr  
965 970 975

Lys Tyr Asn Leu Asp Leu Thr Asn Leu Asn Gln Pro Leu Leu Asp Val  
980 985 990

Asp His Thr Ser Ser Arg Leu Asn Leu Leu Thr Pro Arg His Leu Asn  
995 1000 1005

Gln Lys Gly Lys Ala Leu Pro Leu Ser Ser Ala Glu Lys Arg Lys  
1010 1015 1020

Ala Lys Trp Glu Ser Leu Gln Asn Lys Gln Ile Leu Val Pro Glu  
1025 1030 1035

Leu Cys Ala Ile His Pro Ile Pro Ala Ser Leu Trp Arg Lys Ala  
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Val Cys Leu Pro Ser Ile Leu Tyr Arg Leu His Cys Leu Leu Thr  
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Ala Glu Glu Leu Arg Ala Gln Thr Ala Ser Asp Ala Gly Val Gly  
1070 1075 1080

Val Arg Ser Leu Pro Ala Asp Phe Arg Tyr Pro Asn Leu Asp Phe  
1085 1090 1095

Gly Trp Lys Lys Ser Ile Asp Ser Lys Ser Phe Ile Ser  
1100 1105 1110

Asn Ser Ser Ser Ala Glu Asn Asp Asn Tyr Cys Lys His Ser Thr  
1115 1120 1125

Ile Val Pro Glu Asn Ala Ala His Gln Gly Ala Asn Arg Thr Ser  
1130 1135 1140

Ser Leu Glu Asn His Asp Gln Met Ser Val Asn Cys Arg Thr Leu  
1145 1150 1155

Leu Ser Glu Ser Pro Gly Lys Leu His Val Glu Val Ser Ala Asp  
1160 1165 1170

Leu Thr Ala Ile Asn Gly Leu Ser Tyr Asn Gln Asn Leu Ala Asn  
1175 1180 1185

Gly Ser Tyr Asp Leu Ala Asn Arg Asp Phe Cys Gln Gly Asn Gln  
1190 1195 1200

Leu Asn Tyr Tyr Lys Gln Glu Ile Pro Val Gln Pro Thr Thr Ser  
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Tyr Ser Ile Gln Asn Leu Tyr Ser Tyr Glu Asn Gln Pro Gln Pro  
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Ser Asp Glu Cys Thr Leu Leu Ser Asn Lys Tyr Leu Asp Gly Asn  
1235 1240 1245

Ala Asn Lys Ser Thr Ser Asp Gly Ser Pro Val Met Ala Val Met  
1250 1255 1260

Pro Gly Thr Thr Asp Thr Ile Gln Val Leu Lys Gly Arg Met Asp  
1265 1270 1275

Ser Glu Gln Ser Pro Ser Ile Gly Tyr Ser Ser Arg Thr Leu Gly  
1280 1285 1290

Pro Asn Pro Gly Leu Ile Leu Gln Ala Leu Thr Leu Ser Asn Ala  
1295 1300 1305

Ser Asp Gly Phe Asn Leu Glu Arg Leu Glu Met Leu Gly Asp Ser  
1310 1315 1320

Phe Leu Lys His Ala Ile Thr Thr Tyr Leu Phe Cys Thr Tyr Pro  
1325 1330 1335

Asp Ala His Glu Gly Arg Leu Ser Tyr Met Arg Ser Lys Lys Val  
1340 1345 1350

Ser Asn Cys Asn Leu Tyr Arg Leu Gly Lys Lys Lys Gly Leu Pro  
1355 1360 1365

Ser Arg Met Val Val Ser Ile Phe Asp Pro Pro Val Asn Trp Leu

1370 1375 1380

Pro Pro Gly Tyr Val Val Asn Gln Asp Lys Ser Asn Thr Asp Lys  
1385 1390 1395Trp Glu Lys Asp Glu Met Thr Lys Asp Cys Met Leu Ala Asn Gly  
1400 1405 1410Lys Leu Asp Glu Asp Tyr Glu Glu Glu Asp Glu Glu Glu Ser  
1415 1420 1425Leu Met Trp Arg Ala Pro Lys Glu Glu Ala Asp Tyr Glu Asp Asp  
1430 1435 1440Phe Leu Glu Tyr Asp Gln Glu His Ile Arg Phe Ile Asp Asn Met  
1445 1450 1455Leu Met Gly Ser Gly Ala Phe Val Lys Lys Ile Ser Leu Ser Pro  
1460 1465 1470Phe Ser Thr Thr Asp Ser Ala Tyr Glu Trp Lys Met Pro Lys Lys  
1475 1480 1485Ser Ser Leu Gly Ser Met Pro Phe Ser Ser Asp Phe Glu Asp Phe  
1490 1495 1500Asp Tyr Ser Ser Trp Asp Ala Met Cys Tyr Leu Asp Pro Ser Lys  
1505 1510 1515

Ala Val Glu Glu Asp Asp Phe Val Val Gly Phe Trp Asn Pro Ser  
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Glu Glu Asn Cys Gly Val Asp Thr Gly Lys Gln Ser Ile Ser Tyr  
1535 1540 1545

Asp Leu His Thr Glu Gln Cys Ile Ala Asp Lys Ser Ile Ala Asp  
1550 1555 1560

Cys Val Glu Ala Leu Leu Gly Cys Tyr Leu Thr Ser Cys Gly Glu  
1565 1570 1575

Arg Ala Ala Gln Leu Phe Leu Cys Ser Leu Gly Leu Lys Val Leu  
1580 1585 1590

Pro Val Ile Lys Arg Thr Asp Arg Glu Lys Ala Leu Cys Pro Thr  
1595 1600 1605

Arg Glu Asn Phe Asn Ser Gln Gln Lys Asn Leu Ser Val Ser Cys  
1610 1615 1620

Ala Ala Ala Ser Val Ala Ser Ser Arg Ser Ser Val Leu Lys Asp  
1625 1630 1635

Ser Glu Tyr Gly Cys Leu Lys Ile Pro Pro Arg Cys Met Phe Asp  
1640 1645 1650

His Pro Asp Ala Asp Lys Thr Leu Asn His Leu Ile Ser Gly Phe  
1655 1660 1665

Glu Asn Phe Glu Lys Lys Ile Asn Tyr Arg Phe Lys Asn Lys Ala  
1670 1675 1680

Tyr Leu Leu Gln Ala Phe Thr His Ala Ser Tyr His Tyr Asn Thr  
1685 1690 1695

Ile Thr Asp Cys Tyr Gln Arg Leu Glu Phe Leu Gly Asp Ala Ile  
1700 1705 1710

Leu Asp Tyr Leu Ile Thr Lys His Leu Tyr Glu Asp Pro Arg Gln  
1715 1720 1725

His Ser Pro Gly Val Leu Thr Asp Leu Arg Ser Ala Leu Val Asn  
1730 1735 1740

Asn Thr Ile Phe Ala Ser Leu Ala Val Lys Tyr Asp Tyr His Lys  
1745 1750 1755

Tyr Phe Lys Ala Val Ser Pro Glu Leu Phe His Val Ile Asp Asp  
1760 1765 1770

Phe Val Gln Phe Gln Leu Glu Lys Asn Glu Met Gln Gly Met Asp  
1775 1780 1785

Ser Glu Leu Arg Arg Ser Glu Glu Asp Glu Glu Lys Glu Glu Asp  
1790 1795 1800

Ile Glu Val Pro Lys Ala Met Gly Asp Ile Phe Glu Ser Leu Ala  
1805 1810 1815

Gly Ala Ile Tyr Met Asp Ser Gly Met Ser Leu Glu Thr Val Trp  
1820 1825 1830

Gln Val Tyr Tyr Pro Met Met Arg Pro Leu Ile Glu Lys Phe Ser  
1835 1840 1845

Ala Asn Val Pro Arg Ser Pro Val Arg Glu Leu Leu Glu Met Glu  
1850 1855 1860

Pro Glu Thr Ala Lys Phe Ser Pro Ala Glu Arg Thr Tyr Asp Gly  
1865 1870 1875

Lys Val Arg Val Thr Val Glu Val Val Gly Lys Gly Lys Phe Lys  
1880 1885 1890

Gly Val Gly Arg Ser Tyr Arg Ile Ala Lys Ser Ala Ala Ala Arg  
1895 1900 1905

Arg Ala Leu Arg Ser Leu Lys Ala Asn Gln Pro Gln Val Pro Asn  
1910 1915 1920

Ser

&lt;210&gt; 3

&lt;211&gt; 6750

&lt;212&gt; DNA

&lt;213&gt; Drosophila melanogaster

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(6750)

&lt;400&gt; 3

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Met Ala Phe His Trp Cys Asp Asn Asn Leu His Thr Thr Val Phe Thr

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ccg cgc gac ttt cag gtc gag cta ctg gcc acc gcc tac gag cgg aac 96

Pro Arg Asp Phe Gln Val Glu Leu Leu Ala Thr Ala Tyr Glu Arg Asn

20 25 30

acg att att tgc ctg ggc cat cga agt tcc aag gag ttt ata gcc ctc 144

Thr Ile Ile Cys Leu Gly His Arg Ser Ser Lys Glu Phe Ile Ala Leu

35 40 45

aag ctg ctc cag gag ctg tgc cgt cga gca cgc cga cat ggt cgt gtc 192

Lys Leu Leu Gln Glu Leu Ser Arg Arg Ala Arg Arg His Gly Arg Val

50 55 60

agt gtc tat ctc agt tgc gag gtt ggc acc agc acg gaa cca tgc tcc 240

Ser Val Tyr Leu Ser Cys Glu Val Gly Thr Ser Thr Glu Pro Cys Ser

65 70 75 80

atc tac acg atg ctc acc cac ttg act gac ctg cgg gtc tgg cag gag 288

Ile Tyr Thr Met Leu Thr His Leu Thr Asp Leu Arg Val Trp Gln Glu

85 90 95

cag ccg gat atg caa att ccc ttt gat cat tgc tgg acg gac tat cac 336  
 Gin Pro Asp Met Gln Ile Pro Phe Asp His Cys Trp Thr Asp Tyr His  
 100 105 110

gtt tcc atc cta cgg cca gag gga ttt ctt tat ctg ctc gaa act cgc 384  
 Val Ser Ile Leu Arg Pro Glu Gly Phe Leu Tyr Leu Leu Glu Thr Arg  
 115 120 125

gag ctg ctg ctg agc agc gtc gaa ctg atc gtg ctg gaa gat tgt cat 432  
 Glu Leu Leu Leu Ser Ser Val Glu Leu Ile Val Leu Glu Asp Cys His  
 130 135 140

gac agc gcc gtt tat cag agg ata agg cct ctg ttc gag aat cac att 480  
 Asp Ser Ala Val Tyr Gin Arg Ile Arg Pro Leu Phe Glu Asn His Ile  
 145 150 155 160

atg cca gcg cca ccg gcg gac agg cca cgg att ctc gga ctc gct gga 528  
 Met Pro Ala Pro Pro Ala Asp Arg Pro Arg Ile Leu Gly Leu Ala Gly  
 165 170 175

ccg ctg cac agc gcc gga tgt gag ctg cag caa ctg agc gcc atg ctg 576  
 Pro Leu His Ser Ala Gly Cys Glu Leu Gln Gln Leu Ser Ala Met Leu  
 180 185 190

gcc acc ctg gag cag agt gtg ctt tgc cag atc gag acg gcc agt gat 624  
 Ala Thr Leu Glu Gln Ser Val Leu Cys Gln Ile Glu Thr Ala Ser Asp  
 195 200 205

att gtc acc gtg ttg cgt tac tgt tcc cga ccg cac gaa tac atc gta 672  
 Ile Val Thr Val Leu Arg Tyr Cys Ser Arg Pro His Glu Tyr Ile Val  
 210 215 220

cag tgc gcc ccc ttc gag atg gac gaa ctg tcc ctg gtg ctt gcc gat 720  
 Gln Cys Ala Pro Phe Glu Met Asp Glu Leu Ser Leu Val Leu Ala Asp  
 225 230 235 240

gtg ctc aac aca cac aag tcc ttt tta ttg gac cac cgc tac gat ccc 768

Val Leu Asn Thr His Lys Ser Phe Leu Leu Asp His Arg Tyr Asp Pro

245 250 255

tac gaa atc tac ggc aca gac cag ttt atg gac gaa ctg aaa gac ata 816

Tyr Glu Ile Tyr Gly Thr Asp Gln Phe Met Asp Glu Leu Lys Asp Ile

260 265 270

ccc gat ccc aag gtg gac ccc ctg aac gtc atc aac tca cta ctg gtc 864

Pro Asp Pro Lys Val Asp Pro Leu Asn Val Ile Asn Ser Leu Leu Val

275 280 285

gtg ctg cac gag atg ggt cct tgg tgc acg cag cgg gct gca cat cac 912

Val Leu His Glu Met Gly Pro Trp Cys Thr Gln Arg Ala Ala His His

290 295 300

ttt tac caa tgc aat gag aag tta aag gtg aag acg ccg cac gaa cgt 960

Phe Tyr Gln Cys Asn Glu Lys Leu Lys Val Lys Thr Pro His Glu Arg

305 310 315 320

cac tac ttg ctg tac tgc cta gtg agc acg gcc ctt atc caa ctg tac 1008

His Tyr Leu Leu Tyr Cys Leu Val Ser Thr Ala Leu Ile Gln Leu Tyr

325 330 335

tcc ctc tgc gaa cac gca ttc cat cga cat tta gga agt ggc agc gat 1056

Ser Leu Cys Glu His Ala Phe His Arg His Leu Gly Ser Gly Ser Asp

340 345 350

tca cgc cag acc atc gaa cgc tat tcc agc ccc aag gtg cga cgt ctg 1104

Ser Arg Gln Thr Ile Glu Arg Tyr Ser Ser Pro Lys Val Arg Arg Leu

355 360 365

ttg cag aca ctg agg tgc ttc aag ccg gaa gag gtg cac acc caa gcg 1152

Leu Gln Thr Leu Arg Cys Phe Lys Pro Glu Glu Val His Thr Gln Ala

370 375 380

gac gga ctg cgc aga atg ccg cat cag gtg gat cag gcg gac ttc aat 1200

Asp Gly Leu Arg Arg Met Arg His Gln Val Asp Gln Ala Asp Phe Asn

385 390 395 400

cggtatctcatacgtctgaaagc aag tgc cga atg gtg gat caa atg 1248  
Arg Leu Ser His Thr Leu Glu Ser Lys Cys Arg Met Val Asp Gln Met

405 410 415

gac caa ccg ccg acg gag aca cga gcc ctg gtg gcc act ctt gag cag 1296  
Asp Gln Pro Pro Thr Glu Thr Arg Ala Leu Val Ala Thr Leu Glu Gln

420 425 430

att ctg cac acg aca gag gac agg cag acg aac aga agc gcc gct cgg 1344  
Ile Leu His Thr Thr Glu Asp Arg Gln Thr Asn Arg Ser Ala Ala Arg

435 440 445

gtg act cct act cct act ccc gct cat gcg aag ccg aaa cct agc tct 1392  
Val Thr Pro Thr Pro Ala His Ala Lys Pro Lys Pro Ser Ser

450 455 460

ggt gcc aac act gca caa cca cga act cgt aga cgt gtg tac acc agg 1440  
Gly Ala Asn Thr Ala Gln Pro Arg Thr Arg Arg Arg Val Tyr Thr Arg

465 470 475 480

cgc cac cac ccg gat cac aat gat ggc agc gac acg ctc tgc gca ctg 1488  
Arg His His Arg Asp His Asn Asp Gly Ser Asp Thr Leu Cys Ala Leu

485 490 495

att tac tgc aac cag aac cac acg gct cgc gtg ctc ttt gag ctt cta 1536  
Ile Tyr Cys Asn Gln Asn His Thr Ala Arg Val Leu Phe Glu Leu Leu

500 505 510

gcg gag att agc aga cgt gat ccc gat ctc aag ttc cta cgc tgc cag 1584  
Ala Glu Ile Ser Arg Arg Asp Pro Asp Leu Lys Phe Leu Arg Cys Gln

515 520 525

tac acc acg gac ccg gtg gca gat ccc acc acg gag ccc aaa gag gct 1632  
Tyr Thr Thr Asp Arg Val Ala Asp Pro Thr Thr Glu Pro Lys Glu Ala

530 535 540

gag ttg gag cac cgg cgg cag gaa gag gtg cta aag cgc ttc cgc atg 1680  
 Glu Leu Glu His Arg Arg Gln Glu Glu Val Leu Lys Arg Phe Arg Met  
 545 550 555 560

cat gac tgc aat gtc ctg atc ggt act tcg gtg ctg gaa gag ggc atc 1728  
 His Asp Cys Asn Val Leu Ile Gly Thr Ser Val Leu Glu Glu Ile  
 565 570 575

gat gtg ccc aag tgc aat ttg gtt gtg cgc tgg gat ccg cca acc aca 1776  
 Asp Val Pro Lys Cys Asn Leu Val Val Arg Trp Asp Pro Pro Thr Thr  
 580 585 590

tat cgc agt tac gtt cag tgc aaa ggt cga gcc cgt gct gct cca gcc 1824  
 Tyr Arg Ser Tyr Val Gln Cys Lys Gly Arg Ala Arg Ala Ala Pro Ala  
 595 600 605

tat cat gtc att ctc gtc gct ccg agt tat aaa agc cca act gtg ggg 1872  
 Tyr His Val Ile Leu Val Ala Pro Ser Tyr Lys Ser Pro Thr Val Gly  
 610 615 620

tca gtg cag ctg acc gat cgg agt cat cgg tat att tgc gcg act ggt 1920  
 Ser Val Gln Leu Thr Asp Arg Ser His Arg Tyr Ile Cys Ala Thr Gly  
 625 630 635 640

gat act aca gag gcg gac agc gac tct gat gat tca gcg atg cca aac 1968  
 Asp Thr Thr Glu Ala Asp Ser Asp Ser Asp Ser Ala Met Pro Asn  
 645 650 655

tcg tcc ggc tcg gat ccc tat act ttt ggc acg gca cgc gga acc gtg 2016  
 Ser Ser Gly Ser Asp Pro Tyr Thr Phe Gly Thr Ala Arg Gly Thr Val  
 660 665 670

aag atc ctc aac ccc gaa gtg ttc agt aaa caa cca ccg aca gcg tgc 2064  
 Lys Ile Leu Asn Pro Glu Val Phe Ser Lys Gln Pro Pro Thr Ala Cys  
 675 680 685

gac att aag ctg cag gag atc cag gac gaa ttg cca gcc gca gcg cag 2112  
Asp Ile Lys Leu Gln Glu Ile Gln Asp Glu Leu Pro Ala Ala Ala Gln  
690 695 700

ctg gat acg agc aac tcc agc gac gaa gcc gtc agc atg agt aac acg 2160  
Leu Asp Thr Ser Asn Ser Ser Asp Glu Ala Val Ser Met Ser Asn Thr  
705 710 715 720

tct cca agc gag agc agt aca gaa caa aaa tcc aga cgc ttc cag tgc 2208  
Ser Pro Ser Glu Ser Ser Thr Glu Gln Lys Ser Arg Arg Phe Gln Cys  
725 730 735

gag ctg agc tct tta acg gag cca gaa gac aca agt gat act aca gcc 2256  
Glu Leu Ser Ser Leu Thr Glu Pro Glu Asp Thr Ser Asp Thr Thr Ala  
740 745 750

gaa atc gat act gct cat agt tta gcc agc acc acg aag gac ttg gtg 2304  
Glu Ile Asp Thr Ala His Ser Leu Ala Ser Thr Thr Lys Asp Leu Val  
755 760 765

cat caa atg gca cag tat cgc gaa atc gag cag atg ctg cta tcc aag 2352  
His Gln Met Ala Gln Tyr Arg Glu Ile Glu Gln Met Leu Leu Ser Lys  
770 775 780

tgc gcc aac aca gag ccg ccg gag cag gag cag agt gag ggc gaa cgt 2400  
Cys Ala Asn Thr Glu Pro Pro Glu Gln Glu Gln Ser Glu Ala Glu Arg  
785 790 795 800

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805 810 815

ggc gcc tcc gtg gat ctg ggt tot got ata gct ttg gtc aac aag tac 2496  
Gly Ala Ser Val Asp Leu Gly Ser Ala Ile Ala Leu Val Asn Lys Tyr  
820 825 830

tgc gcc cga ctg cca agc gac acg ttc acc aag ttg acg gcg ttg tgg 2544

Cys Ala Arg Leu Pro Ser Asp Thr Phe Thr Lys Leu Thr Ala Leu Trp

835                    840                    845

cgc tgc acc cga aac gaa agg gct gga gtg acc ctg ttt cag tac aca    2592

Arg Cys Thr Arg Asn Glu Arg Ala Gly Val Thr Leu Phe Gln Tyr Thr

850                    855                    860

ctc cgt ctg ccc atc aac tcg cca ttg aag cat gac att gtg ggt ctt    2640

Leu Arg Leu Pro Ile Asn Ser Pro Leu Lys His Asp Ile Val Gly Leu

865                    870                    875                    880

ccg atg cca actcaa aca ttg gcc cgc cga ctg gct gcc ttg cag gct    2688

Pro Met Pro Thr Gln Thr Leu Ala Arg Arg Leu Ala Ala Leu Gln Ala

885                    890                    895

tgc gtg gaa ctg cac agg atc ggt gag tta gac gat cag ttg cag cct    2736

Cys Val Glu Leu His Arg Ile Gly Glu Leu Asp Asp Gln Leu Gln Pro

900                    905                    910

atc ggc aag gag gga ttg cgt gcc ctg gag ccg gac tgg gag tgc ttt    2784

Ile Gly Lys Glu Gly Phe Arg Ala Leu Glu Pro Asp Trp Glu Cys Phe

915                    920                    925

gaa ctg gag cca gag gac gaa cag att gtg cag cta agc gat gaa cca    2832

Glu Leu Glu Pro Glu Asp Glu Gln Ile Val Gln Leu Ser Asp Glu Pro

930                    935                    940

cgt ccg gga aca acg aag cgt cgt cag tac tat tac aaa cgc att gca    2880

Arg Pro Gly Thr Thr Lys Arg Arg Gln Tyr Tyr Tyr Lys Arg Ile Ala

945                    950                    955                    960

tcc gaa ttg tgc gat tgc cgt ccc gtt gcc gga ggc cca tgc tat ttg    2928

Ser Glu Phe Cys Asp Cys Arg Pro Val Ala Gly Ala Pro Cys Tyr Leu

965                    970                    975

tac ttg atc caa ctg acg ctc caa tgg ccg att ccc gaa gag caa aac    2976

Tyr Phe Ile Gln Leu Thr Leu Gln Cys Pro Ile Pro Glu Glu Gln Asn

980 985 990

acg cgg gga cgc aag att tat ccg ccc gaa gat ggc cag cag gga ttc 3024  
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995 1000 1005

ggc att cta acc acc aaa cgg ata ccc aag ctg agt gct ttc tcg 3069  
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1010 1015 1020

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 Ile Phe Thr Arg Ser Gly Glu Val Lys Val Ser Leu Glu Leu Ala

1025 1030 1035

aag gaa cgc gtg att cta act agc gaa caa ata gtc tgc atc aac 3159  
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1040 1045 1050

gga ttt tta aac tac acg ttc acc aat gta ctg cgt ttg caa aag 3204  
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1055 1060 1065

ttt ctg atg ctc ttc gat ccg gac tcc acg gaa aat tgt gta ttc 3249  
 Phe Leu Met Leu Phe Asp Pro Asp Ser Thr Glu Asn Cys Val Phe

1070 1075 1080

att gtg ccc acc gtg aag gca cca gct ggc ggc aag cac atc gac 3294  
 Ile Val Pro Thr Val Lys Ala Pro Ala Gly Gly Lys His Ile Asp

1085 1090 1095

tgg cag ttt ctg gag ctg atc caa ggc aat gga aat aca atg cca 3339  
 Trp Gln Phe Leu Glu Leu Ile Gln Ala Asn Gly Asn Thr Met Pro

1100 1105 1110

cgg gca gtg ccc gat gag gag cgc cag ggc cag ccg ttt gat ccg 3384  
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1115 1120 1125

caa cgc ttc cag gat gcc gtc gtt atg ccg tgg tat cgc aac cag 3429  
Gln Arg Phe Gln Asp Ala Val Val Met Pro Trp Tyr Arg Asn Gln  
1130 1135 1140

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Asp Gln Pro Gln Tyr Phe Tyr Val Ala Glu Ile Cys Pro His Leu  
1145 1150 1155

tcc cca ctc acg tgc ttc cct ggt gac aac tac cgc acg ttc aag 3519  
Ser Pro Leu Ser Cys Phe Pro Gly Asp Asn Tyr Arg Thr Phe Lys  
1160 1165 1170

cac tac tac ctc gtc aag tat ggt ctg acc ata cag aat acc tcg 3564  
His Tyr Tyr Leu Val Lys Tyr Gly Leu Thr Ile Gln Asn Thr Ser  
1175 1180 1185

cag ccg cta ttg gac gtg gat cac acc agt gcg cgg tta aac ttc 3609  
Gln Pro Leu Leu Asp Val Asp His Thr Ser Ala Arg Leu Asn Phe  
1190 1195 1200

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Leu Thr Pro Arg Tyr Val Asn Arg Lys Gly Val Ala Leu Pro Thr  
1205 1210 1215

agt fcg gag gag aca aag cgg gca aag cgc gag aat ctc gaa cag 3699  
Ser Ser Glu Glu Thr Lys Arg Ala Lys Arg Glu Asn Leu Glu Gln  
1220 1225 1230

aag cag atc ctt gtg cca gag ctc tgc act gtg cat cca ttc ccc 3744  
Lys Gin Ile Leu Val Pro Glu Leu Cys Thr Val His Pro Phe Pro  
1235 1240 1245

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Ala Ser Leu Trp Arg Thr Ala Val Cys Leu Pro Cys Ile Leu Tyr  
1250 1255 1260

cgc ata aat ggt ctt cta ttg gcc gac gat att cgg aaa cag gtt 3834  
 Arg Ile Asn Gly Leu Leu Leu Ala Asp Asp Ile Arg Lys Gln Val  
 1265 1270 1275

tct gcg gat ctg ggg ctg gga agg caa cag atc gaa gat gag gat 3879  
 Ser Ala Asp Leu Gly Leu Gly Arg Gln Gln Ile Glu Asp Glu Asp  
 1280 1285 1290

ttc gag tgg ccc atg ctg gac ttt ggg tgg agt cta tcg gag gtg 3924  
 Phe Glu Trp Pro Met Leu Asp Phe Gly Trp Ser Leu Ser Glu Val  
 1295 1300 1305

ctc aag aaa tcg cgg gag tcc aaa caa aag gag tcc ctt aag gat 3969  
 Leu Lys Lys Ser Arg Glu Ser Lys Gln Lys Glu Ser Leu Lys Asp  
 1310 1315 1320

gat act att aat ggc aaa gac tta gct gat gtt gaa aag aaa ccg 4014  
 Asp Thr Ile Asn Gly Lys Asp Leu Ala Asp Val Glu Lys Lys Pro  
 1325 1330 1335

act agc gag gag acc caa cta gat aag gat tca aaa gac gat aag 4059  
 Thr Ser Glu Glu Thr Gln Leu Asp Lys Asp Ser Lys Asp Asp Lys  
 1340 1345 1350

gtt gag aaa agt gct att gaa cta atc att gag gga gag gag aag 4104  
 Val Glu Lys Ser Ala Ile Glu Leu Ile Ile Glu Gly Glu Glu Lys  
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Asn Lys Gln Gly Gly Lys Gly Lys Ala Lys Gly Pro Ala Lys Pro

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Thr Phe Asn Tyr Tyr Asp Ser Asp Asn Ser Leu Gly Ser Ser Tyr

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Val Leu Asn Thr His Lys Ser Phe Leu Leu Asp His Arg Tyr Asp Pro

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625 630 635 640

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645 650 655

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660 665 670

Lys Ile Leu Asn Pro Glu Val Phe Ser Lys Gln Pro Pro Thr Ala Cys  
675 680 685

Asp Ile Lys Leu Gln Glu Ile Gln Asp Glu Leu Pro Ala Ala Ala Gln

690                695                700

Leu Asp Thr Ser Asn Ser Ser Asp Glu Ala Val Ser Met Ser Asn Thr

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Ser Pro Ser Glu Ser Ser Thr Glu Gln Lys Ser Arg Arg Phe Gln Cys

725                730                735

Glu Leu Ser Ser Leu Thr Glu Pro Glu Asp Thr Ser Asp Thr Thr Ala

740                745                750

Glu Ile Asp Thr Ala His Ser Leu Ala Ser Thr Thr Lys Asp Leu Val

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His Gln Met Ala Gln Tyr Arg Glu Ile Glu Gln Met Leu Leu Ser Lys

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Pro Met Pro Thr Gln Thr Leu Ala Arg Arg Leu Ala Ala Leu Gln Ala

885 890 895

Cys Val Glu Leu His Arg Ile Gly Glu Leu Asp Asp Gln Leu Gln Pro

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Tyr Phe Ile Gln Leu Thr Leu Gln Cys Pro Ile Pro Glu Glu Gln Asn

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2245

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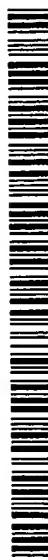
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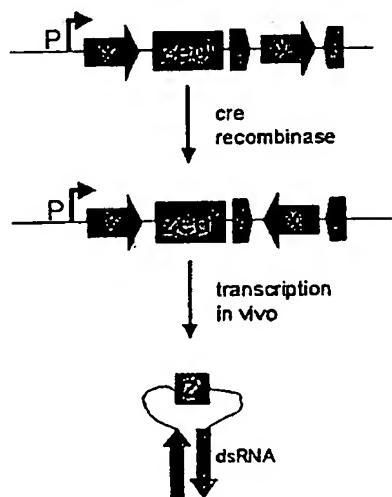
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS AND COMPOSITIONS FOR RNA INTERFERENCE



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(57) Abstract: The present invention provides methods for attenuating gene expression in a cell using gene-targeted double stranded RNA (dsRNA). The dsRNA contains a nucleotide sequence that hybridizes under physiologic conditions of the cell to the nucleotide sequence of at least a portion of the gene to be inhibited (the "target" gene).

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/08435

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 7 C12N15/11 C12N15/63 C12Q1/68 A61K31/7105

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, CAB Data, SEQUENCE SEARCH, BIOSIS, EPO-Internal

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TUSCHL THOMAS ET AL: "Targeted mRNA degradation by double-stranded RNA in vitro." GENES & DEVELOPMENT, vol. 13, no. 24, 15 December 1999 (1999-12-15), pages 3191-3197, XP002183118 ISSN: 0890-9369 the whole document ----	1,9, 12-15
A	WO 99 32619 A (CARNEGIE INST OF WASHINGTON ;MONTGOMERY MARY K (US); FIRE ANDREW () 1 July 1999 (1999-07-01) the whole document ----	-/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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\*&\* document member of the same patent family

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/08435

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 01 29058 A (CARNEGIE INST OF WASHINGTON ;UNIV MASSACHUSETTS (US)) 26 April 2001 (2001-04-26) claims 1-16 ---	1-5, 9, 12-15
P, X	WO 00 44914 A (FARRELL MICHAEL J ;LI YIN XIONG (US); KIRBY MARGARET L (US); MEDIC) 3 August 2000 (2000-08-03) claims 1-61 ---	1, 9, 12-15
P, X	BERNSTEIN EMILY ET AL: "Role for a bidentate ribonuclease in the initiation step of RNA interference." NATURE (LONDON), vol. 409, no. 6818, 18 January 2001 (2001-01-18), pages 363-366, XP002183119 ISSN: 0028-0836 the whole document ---	1-15
T	HAMMOND SCOTT M ET AL: "Argonaute2, a link between genetic and biochemical analyses of RNAi." SCIENCE (WASHINGTON D C), vol. 293, no. 5532, 2001, pages 1146-1150, XP002183120 ISSN: 0036-8075 figure 3 ---	
T	MOSS ERIC G: "RNA interference: It's a small RNA world." CURRENT BIOLOGY, vol. 11, no. 19, 2001, pages R772-R775, XP002183121 ISSN: 0960-9822 page R773, right-hand column, line 8 -page R774, right-hand column, line 23 ---	
T	AMBROS VICTOR: "Dicing up RNAs." SCIENCE (WASHINGTON D C), vol. 293, no. 5531, 3 August 2001 (2001-08-03), pages 811-813, XP002183122 ISSN: 0036-8075 the whole document ---	
		-/-

## INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/US 01/08435

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CAPLEN N J ET AL: "dsRNA-mediated gene silencing in cultured Drosophila cells: a tissue culture model for the analysis of RNA interference" GENE, ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, NL, vol. 252, no. 1-2, 11 July 2000 (2000-07-11), pages 95-105, XP004210158 ISSN: 0378-1119 the whole document -----	1,9, 12-15
P,A	HAMMOND SCOTT M ET AL: "An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells." NATURE (LONDON), vol. 404, no. 6775, 16 March 2000 (2000-03-16), pages 293-296, XP002183123 ISSN: 0028-0836 the whole document -----	

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

Continuation of Box I.1

Although claims (2,4-15)-partially (and as far as in vivo methods are concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

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Continuation of Box I.1

Although claims 18 and 19 could be, at least be partially be considered as scheme, rule and method for doing business, Rule 39.1(iii) PCT, the search has been carried out as far as possible in our systematic documentation.

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Continuation of Box I.2

Claims Nos.: 17

Present claim 17 relate to a method of formulating a pharmaceutical preparation including one or more agents identified by an assay, without giving a true technical characterization. Moreover no such compounds are defined in the application. In consequence, the scope of said claims is ambiguous and vague, and their subject-matter is not sufficiently disclosed and supported (Art. 5 and 6 PCT). No search can be carried out for such purely speculative claims whose wording is, in fact, a mere recitation of the results to be achieved.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 01/08435

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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